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Development of methods for multi-allergen detection and quantification in food by mass spectrometry

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DEVELOPMENT OF METHODS FOR MULTI-ALLERGEN DETECTION AND QUANTIFICATION IN FOOD BY MASS SPECTROMETRY

Original dissertation presented by **Melanie PLANQUE** in order to obtain the degree of Doctor in Sciences

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Summary

Worldwide, food allergy is a growing health problem which can provoke mild to severe symptoms responsible for several hundred of deaths each year. To avoid allergic reactions, the allergic population should avoid food products containing the causative allergenic proteins. Currently, however, as most food suppliers are not in a position to completely guarantee allergen-free products, they abusively use precautionary allergen labeling of the type “may contain…”. This leads to a lack of clear information for the allergic population.

For several years, laboratories have been working on the development of reliable and efficient analytical methods for detecting traces of allergens in food products. The diversity of food products and industrial processes makes this quite a challenge. The enzyme-linked immunosorbent assay is the method most widely used for detecting and quantifying proteins of allergenic foods. However, after the thermal process, protein recognition is decreased or even compromised, and complementary methods must be developed.

In the framework of this thesis, we have developed a single method based on ultra-high-performance liquid chromatography coupled to tandem mass spectrometry (UHPLC-MS/MS) for the detection and quantification of several allergenic proteins in processed food products. We have focused on the detection of ten allergenic foods: milk, egg, soy, peanut, and tree nuts (almond, hazelnut, walnut, pecan nut, cashew, and pistachio) in four processed or complex matrices (cookie (180°C – 18 min), sauce (95°C - 45 min), chocolate (tannins), and ice cream (fat)).

The developed method allows the reliable detection of proteins from ten allergenic foods in a wide variety of food products with high sensitivity and specificity that could ensure the protection of the allergic population. Our allergen detection tests performed on processed and unprocessed food products provide a basis for reflection and enables us to make some recommendations (regarding, for example, the selection of marker peptides and the acceptance criteria defined for positive and negative samples) for the development of a routine UHPLC-MS/MS-based allergen detection method. We have also developed a quantification method combining two strategies: standard addition and use of labeled peptides. It ensures, with good recovery, both the detection of allergens at the determined sensitivity and reliable quantification of allergens in all kinds of foodstuffs. To the best of our knowledge, this routine UHPLC-MS/MS is the first to detect processed allergenic proteins with such high sensitivity in several food products within a day. The sensitivity of the developed method applied, to processed foods constitutes a significant step forward towards protection of the allergic population through improved food labeling.

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ABBREVIATIONS

A

AGEP: Acute Generalized Exanthematous Pustulosis

Ana o: *Anacardium occidentale*

APCs: Antigen-presenting cells

AOAC International: American Organization of Analytical Chemists International

APT: Atopy Patch Test

Ara h: *Arachis hypogaea*

B

BCA: BiCinchoninic Acid assay

Bet v: *Betula verrucosa*

Ber e: *Bertholletia excela*

BLAST: Basic Local Alignment Search Tool

Bos d: *Bos domesticus*

BSA : Bovine serum albumin

C

C: constant

CAC: Codex Alimentarius Commission

Car i: *Carya illinoinensis*

$CD4^+$: Cluster of Differentiation 4

$CD8^+$: Cluster of Differentiation 8

CDR: Complementary Determining Regions

CFIA: Canadian Food Inspection Agency

CTL: Cytotoxic T Lymphocytes

Cor a: *Corylus avellana*

CXCL-8: CXC- Chemokine Ligand 8

D

DBPCFC: Double-Blind Placebo Controlled Food Challenge

DC: Dendritic Cells

DMSO: DiMethyl SulfOxide

DNA: Deoxyribonucleic acid

DTT: 1 4-DiThioThreitol

E

EAACI: European Academy of Allergology and Clinical Immunology

ECP: Eosinophil Cationic Protein

ED: Eliciting Dose

EDN: Eosinophil-Derived Neurotoxin

EFSA: European Food Safety Authority agency

ELISA: Enzyme-Linked ImmunoSorbent Assay

EoE: Eosinophilic Esophagitis

EPIT: EPicutaneous ImmunoTherapy

EPO: Eosinophil PerOxidase

F FAAN: Food Allergy and Anaphylaxis Network

Fab: Fragment antigen binding

FAHF: Food Allergy Herbal Formula

FALCPA: Food Allergen Labeling and Consumer Protection Actions

FAO: Food and Agricultural Organization

FARRP: Food Allergy Research and Resource Programme

FASFC: Federal Agency for the Safety of the Food Chain

Fc: Fragment crystallizable

FcεRI: High-affinity immunoglobulin ε Fragment crystallizable region Receptor I

FDA: Food and Drug Administration

FEIA: Fluorescence Enzyme Immuno Assay

FOXP3: FOrkhead boX P3

G Gal d : *Gallus domesticus*

GLUT 5: Glucose transport protein 5

GLUT 2: Glucose transport protein 2

Gly m: *Glycine max*

GM-CSF: Granulocyte Macrophage – Colony Stimulating Factor

H H: Heavy

HACCP: Hazard Analysis Critical Control Point approach

HLA: Human Leucocyte Antigen

HR2: Histamine Receptor 2

HRMS: High Resolution Mass Spectrometry

I IAA: Iodoacetamide

IAC: ImmunoAffinity Columns

IFN-γ: Interferon-γ

Ig: Immunoglobulin

IL: Interleukin

Ile, I: Isoleucine

IS: Internal Standard

IUIS : International Union of Immunological Societies

J Jug n : *Juglans nigra*

Jug r : *Juglans regia*

L Leu, L: Leucine

LTC4: Leukotriene C4

LTD4: Leukotriene D4

LTE4: Leukotriene E4

LOAL: Low Observable Adverse Effect Levels

LOD: Limit Of Detection

LOQ: Limit Of Quantification

M m: mass

MALDI: Matrix-Assisted Laser Desorption Ionization

MBP: Major Basic Protein

MDL: Method Detection Limit

MHC II: Major Histocompatibility Complex class II

MLQ: Minimum Level of Quantitation

MRM: Multiple Reaction Monitoring

MS: Mass spectrometry

N NIST: National Institute of Standards and Technology

NK: Natural Killer cells

NLR: NOD-like receptors

NOAEL : No Observable Adverse Effect Levels

NOD: Nucleotide binding Oligomerization Domain

NVWA: Nederlandse Voedsel- en WarenAutoriteit (Netherlands food and consumer product safety authority)

O OAS: Oral Allergy Symptoms

OFC: Oral Food Challenge

OIT: Oral ImmunoTherapy

P PAF: Platelet-Activating Factor

PAL: Precautionary Allergen Labeling

PAMP: Pathogen-Associated Molecular Pattern

PCR: Polymerase Chain Reaction

PD1: Programmed cell death 1

PGD2: Prostaglandin D2

Phe, F: Phenylalanine

pI: Isoelectric point

Pis v: *Pistachio vera*

PMN: PolyMorphonuclear Neutrophil

PRR: Pathogen Recognition Receptor

Pru du: *Prunus dulcis*

R RASFF: Rapid Alert System for Food and Feed

RAST: RadioAllergoSorbent Test

RIG: Retinoic acid–Inducible Gene

RLR: RIG-I-Like Receptor

RT: Retention time

S S/N: Signal-to-Noise

SD: Standard Deviation

SEC: Size Exclusion Chromatography

sIgE: Serum-specific IgE testing

SLIT: SubLingual ImmunoTherapy

SMPR: Standard Method Performance Requirements

SPE: Solid Phase Extraction

SPT: Skin Prick Test

STAT6: Signal Ttransducer and Activator of Transcription 6

T TCEP: Tris(2-carboxyethyl)phosphine

TCR: T-Cell Receptor

TGF: Transforming Growth Factor

TLR: Toll-Like Receptor

TNF-α: Tumor-necrosis factor-alpha

T_H2: T helper 2 cells

TOF: Time Of Flight

Treg: T regulatory cells

Tris: Tris(hydroxymethyl)aminomethane

Trp, W: Tryptophan

TSP 1: Thrombospondin 1

U UHPLC: Ultra-High Performance Liquid Chromatography

V Val, V: Valine

VITAL: Voluntary Incidental Trace Allergen Labeling

W WHO: World Health Organization

Z z: Charge

INTRODUCTION

A food allergy is a pathological disorder of the immune system, triggered by ingestion of an antigen called an allergen (Nadeau et al., 2012; Yu et al., 2016). Such disorders affect 5% of adults and at least 8% of children in developed countries (Sicherer et al., 2014). Food allergy is now a major public health problem, ranked by the World Health Organization (WHO) as the sixth human health problem (Asero et al., 2007; Kumar et al., 2012). Food allergies have been increasing over the last decades with clinical symptoms ranging from mild (e.g. eczema, skin allergy) to severe (anaphylaxis) (Branum et al., 2008; Fiocchi et al., 2017). In the United States, 30,000 people are treated in hospital yearly for anaphylaxis after ingestion of a food allergen, and for some 150 individuals/year, the anaphylaxis reaction is lethal (Kumar et al., 2012; Verma et al., 2013).

Allergic reactions can be triggered by more than 170 food ingredients, but proteins from 8 allergenic foods are responsible for over 90% of all allergic reactions in developed countries: milk, egg, peanut, tree nuts, shellfish, fish, wheat, and soy (Boye, 2012).

To avoid an allergic reaction, the allergenic food must be totally excluded from the diet for the most sensitive individuals. This implies that the labeling of foodstuffs must be very reliable. In 1995, the European Union legislation required that all ingredients present at concentrations higher than 25% had to be mentioned on the label (Bruijnzeel-Koomen et al., 1995; Eigenmann, 2001). This legislation has evolved considerably (Regulation No 1169/2011), now requiring that 14 allergenic foods and their derived products be declared on the label when they are incorporated as ingredients: milk, eggs, cereals containing gluten (wheat, rye, and barley), fish, crustaceans, mollusks, tree nuts (almonds, hazelnuts, walnuts, cashews, pecan nuts, Brazil nuts, pistachio, macadamia), soy, peanuts, sesame, lupine, mustard, celery, and sulfur dioxide (sulfites) (Regulation No. 1169/2011, 2011). Over the past few decades, considerable improvements have been achieved in food safety and in declaring the presence of food allergens on food labels, however the presence of cross contamination during food production is still widespread (Regulation 178/2002/EC, 2002). For this reason, the current situation still cannot guarantee the total safety of the food-allergic population.

The target allergens of the present project were milk, egg, soy, peanut, and tree nut. The present introduction first describes their characteristics before addressing adverse food reactions (section 1), the regulations and labeling requirements applicable to food allergens (section 2), and finally the detection and quantification of food allergen peptides by mass spectrometry (section 3).

I Characteristics of food allergens

The International Union of Immunology Societies (IUIS) defines an allergen, except the sulfites, as a protein responsible for an allergic reaction in at least 5 individuals (Shah et al., 2012). Milk, eggs, and peanuts are responsible for 80% of allergic reactions during childhood, while shellfish, peanuts, tree nuts, and fish allergies are the most prevalent in adulthood.

Resolution of some allergies developed during infancy (allergies to milk, egg, wheat, and soy) can be expected during childhood. For example, 85% of children suffering from cow milk allergies are able to consume milk by age 3, while most patients with peanut, tree nut, and seafood allergies will have a lifelong allergic disease. For example, only 20% of peanut-allergic patients outgrow their allergy (Skolnick et al., 2001; Vickery et al., 2011).

To each allergen is attributed an international code, provided by the IUIS and the Allergen Nomenclature Subcommittee of the WHO. This code contains the first three letters of the genus, followed by the first letter of the taxonomic name of the species and by a number referring to the chronological order of discovery (e.g. casein from *Bos domesticus* is abbreviated as "Bos d 8") (Chapman, 2008; Restani et al., 2009; Kumar et al., 2012).

After ingestion, proteins undergo proteolysis by various enzymes present in saliva, stomach juice, and pancreatic secretions (Vickery et al., 2011). Yet, proteins from allergens belonging to a first class (e.g. milk, egg, peanut, soy, tree nut allergens…) have chemical structures and biological properties that keep them stable upon exposure to acidic conditions, proteases, and heat (Vickery et al., 2011; Shah et al., 2012).

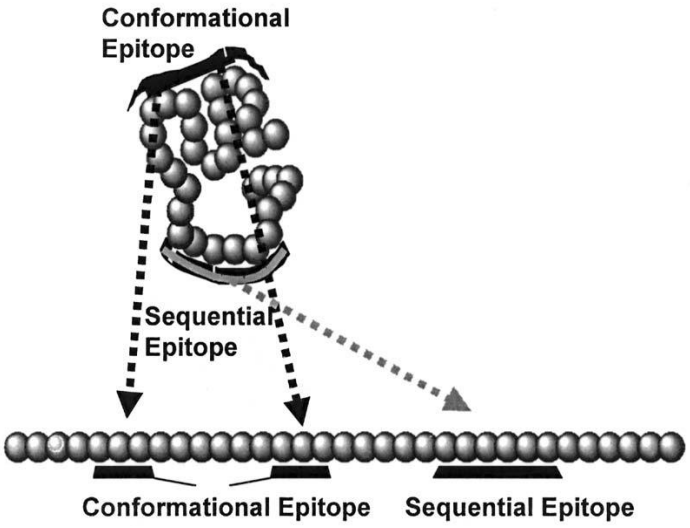


Figure 1: *During thermal processing, conformational epitopes are lost, while sequential epitopes are not affected. This influences the allergenicity of the protein (from* (Sampson, 2004)*).*

Some allergenic proteins, such as ovalbumin and αS1-casein, are considered major allergens because at least 50% of patients in whom the relevant foodstuff triggers an allergic reaction produce specific IgEs against epitopes of these proteins. Other proteins, such as egg yolk proteins (apovitellenin, vitellogenin), are considered minor allergens, even though they can cause severe allergic reactions (Nollet et al., 2011). Immunoglobulin-binding (Ig-binding) epitopes can be linear (continuous) or conformational (discontinuous) (Robotham et al., 2010) (**Figure 1**).

In the next paragraphs, the known allergenic epitopes of major allergenic proteins in milk, egg, peanut, soy, and tree nut are listed.

I.I Milk allergens

Milk allergy is the most prevalent allergy during childhood, affecting between 1.6 and 2.8% of children below the age of 2 (Natale et al., 2004). Milk also ranks third as the cause of fatal or near-fatal allergic reactions, accounting for 8 to 15% of cases (Cianferoni et al., 2012).

Cow milk contains 3 – 3.5% (w/w) protein. Two groups of milk proteins are distinguished: caseins (80%) and whey proteins (20%). Caseins and whey proteins can easily be separated by acidic precipitation at pH 4.6 with hydrochloric acid (Wal, 1998). Caseins (Bos d 8) include α_{S1}-casein (32% of all milk proteins) α_{S2}-casein (10%), β-casein (28%), and κ-casein (10%). Hydrolysis of β-casein generates three γ-caseins (γ1-, γ2-, and γ3-casein) (Restani et al., 2009). Whey proteins include α-lactalbumin (Bos d 4) (5%), β-lactoglobulin (Bos d 5) (10%), bovine serum albumin BSA (Bos d 6) (1%), immunoglobulins (Bos d 7) (3%), and traces of lactoferrin (Nollet et al., 2011). Most patients suffering from cow milk allergy are sensitized to several cow milk proteins (Wal, 2001), and each protein contains several allergenic epitopes (Crittenden et al., 2005). Cow milk contains more than 30 allergenic proteins, but only two are considered major allergens: α_{S1}-casein and β-lactoglobulin.

α_{S1}-casein (Bos d 8) is a 23.6-kDa protein, with a dipolar-type structure (Wal, 2001; Nollet et al., 2011). It consists of 199 amino acids and includes 8 phosphorylated serine residues, a highly negatively charged region, and 45% hydrophobic residues. In a study, the sera of all patients allergic to this protein which shown to recognize three peptides: [aa 19-30], [aa 93-98]), and [aa 141-150], while peptides [aa 50-70], [aa 125-134], [aa 172-183], and [aa 189-198] are recognized by only some patient sera (Spuergin et al., 1996). The fact that some epitopes are recognized by only a low percentage of patient sera makes it hard to treat the allergic population with modified peptides and proteins (section I).

β-lactoglobulin (Bos d 5) is a 36-kDa dimeric protein composed of 162 amino acids, with 2 disulfide bonds and one free cysteine (Wal, 2001). Sera from people allergic to Bos d 5 break down as

follows: 89% reacting with the three peptides [aa 41-60], [aa 102-124] and [aa 149-162], 52% reacting with the four peptides [aa 1-8], [aa 25-40], [aa 21-40] and [aa 92-100], 40% reacting with the two peptides [aa 9-14] and [aa 84-92]. Furthermore, some 28% of patient sera additionally recognize the two peptides, [aa 125-135] and [aa 78-83]. All in all, more than 50% of sera isolated from patients allergic to Bos d 5 recognize at least 6 peptide fragments of β-lactoglobulin (Selo et al., 1999).

Milk-allergic children must thus vary their diet. Soy milk can be a substitute, although 30-40% of infants with cow milk allergy will also develop an allergy to soy formula (Bruijnzeel-Koomen et al., 1995).

I.II Egg allergens

Eggs are the second cause of food allergy in infants, affecting 0.5 to 2.5% of children (Tan et al., 2014). By 5 years of age, 80% of children with egg hypersensitivity are able to consume eggs again. More generally, egg is one of the most prevalent allergy-causing food allergens (Mine et al., 2004). An egg consists of shell (8-11%), white (56-61%), and yolk (27-32%) (Percentage of weight). Egg white contains 10% proteins and 88% water, while egg yolk contains 50% water, 34% lipids, and 16% proteins. In egg white from *Gallus domesticus*, four major allergens are found: ovomucoid (Gal d 1), ovalbumin (Gal d 2), ovotransferrin (Gal d 3), and lysozyme (Gal d 4) (Mine et al., 2004). Egg yolk contains only minor allergens such as α-livetin (Gal d 5), phosvitin, and apovitellenins I and VI (Anet et al., 1985; Walsh et al., 2005).

Ovomucoid (Gal d 1) is a 28-kDa glycoprotein composed of 186 amino acids and containing 20-25% carbohydrates and 5 disulfide bonds. It is a trypsin inhibitor and represents 11% of the total egg-white protein content (Lineweaver et al., 1947; Bernhisel-Broadbent et al., 1994). It is resistant to enzymatic digestion and relatively stable to thermal processing (Mine et al., 2004; Järvinen et al., 2007). Four major IgE epitopes, corresponding to peptides [aa 1-10], [aa 9-20], [aa 47-56], and [aa 113-124], have been identified (Järvinen et al., 2007).

Ovalbumin (Gal d 2) represents 54% of the total egg-white protein content and is a monomeric phosphoglycoprotein with a molecular weight of 44.5 kDa. It comprises 385 amino acids (Bernhisel-Broadbent et al., 1994; Mine et al., 2004). Five epitopes recognized by specific IgEs have been identified: [aa 38-49], [aa 95-102], [aa 191-200], [aa 243-248], and [aa 251-260] (Mine et al., 2003).

Ovotransferrin or **conalbumin** (Gal d 3) is a 76-kDa protein with 686 amino acids, 12 disulfide bonds, and 2.6% carbohydrate moieties (Mine et al., 2004). Identified as bacterium-inhibiting,

ovotransferrin is a heat-labile allergen representing 12% of the egg-white protein content (Mine, 1995; Caubet et al., 2011).

Lysozyme (Gal d 4) is a single, 14-kDa polypeptide chain with 129 amino acids and 4 disulfide bonds (Mine et al., 2004). It is a glycosidase representing 3.4% of the total egg-white protein content. This protein is commonly used for its antibacterial properties, as a preservative in pharmaceutical and food products (Caubet et al., 2011).

I.III Peanut allergens

Peanuts are the most common cause of food allergy in people after the age of 4 (Burks et al., 1991; Shah et al., 2012). Currently, about 1% of the population suffers from peanut allergy, and few affected children outgrow their allergy by the time they reach adulthood (Keet et al., 2007; Skripak et al., 2008). Peanut is also the allergen most commonly involved in anaphylactic shock, accounting for 24 to 30% of all anaphylactic reactions (Hourihane, 1997) and 67% of lethal anaphylactic reactions (Bock et al., 2001).

To date, sixteen peanut (*Arachis hypogaea*) allergens have been identified and listed by the WHO/IUIS: Ara h1 to Ara h17. Of these, only Ara h1, Ara h2, and Ara h3 are viewed as major allergens (Sicherer, 2011). Ara h4, Ara h5, Ara h6, and Ara h7 are minor allergens (Naganawa et al., 2008).

Ara h1 (7S globulin) forms a stable, 64.5-kDa homotrimer complex belonging to the vicilin-like seed storage protein family. Its subunits are held together by hydrophobic interactions at the monomer interfaces, and the complex has no disulfide bonds (Zhang et al., 2017). Ara h1 is recognized by serum IgE from more than 90% of peanut-allergic patients (Maleki et al., 2000 a; Husain et al., 2013). Three immunodominant epitopes have been identified: [aa 409-418], [aa 498-507], and [aa 525-534] (Chruszcz et al., 2011).

Ara h2 (2S globulin) is a 17.5-kDa monomeric protein with 4 disulfide bonds. It is a member of the conglutin family of seed storage proteins (Verma et al., 2013). Three immunodominant epitopes are recognized by all sera prepared from allergic patients: [aa 27-36], [aa 57-66], and [aa 65-74] (Burks et al., 1998).

Ara h3 (11S globulin) is a glycinin-like seed storage protein composed of several polypeptides ranging from 14 to 45 kDa. It includes one acidic and several basic subunits (Koppelman et al., 2004; Sicherer et al., 2009). Between 45 and 95% of IgEs from allergic patient sera recognize Ara h3. Consequently, Ara h3 is viewed in several studies as a minor allergen (Restani et al., 2009; Husain et

al., 2013). To the best of our knowledge, a single major epitope of Ara h3 has been identified [aa 279–293] (Koppelman et al., 2003).

I.IV Soy allergens

Soy allergy affects only 0.2 to 0.3% of the population and triggers mostly mild reactions. In some circumstances, however, it can cause life-threatening reactions (Amnuaycheewa et al., 2010; Verma et al., 2013). A total of 28 proteins have been recognized by sera from soy-allergic patients (Cordle, 2004; Kattan et al., 2011). In 2008, the Food Allergy Research and Resource Program (FARRP) listed 33 IgE-binding sites recognized by soy-allergic patients (Amnuaycheewa et al., 2010). The main allergens of soybean (*Glycine max)* are Gly m Bd 28K, Gly m Bd 30K, and Gly m Bd 60K (Samoto et al., 1997; L'Hocine et al., 2007).

Gly m Bd 28K is a 26-kDa vicilin-like glycoprotein composed of 473 amino acids (L'Hocine et al., 2007). Gly mBd 28K is a minor component of soy but is designated as a major allergen (Ogawa et al., 2000).

Gly m Bd 30K is the main soy allergen, with a molecular weight of 34 kDa. It is the most strongly and frequently allergen recognized by IgEs of allergic patients (Samoto et al., 1997). Gly m Bd 30K is recognized by 65% of sera and possesses five immunodominant epitopes, epitopes recognized by most patient sera: [aa 3-12], [aa 100-110], [aa 229-238], [aa 299-308], and [aa 331-340] (Ogawa et al., 2000; Verma et al., 2013). The glycosylation site of Gly m Bd 30K is located on an Asn residue.

Gly m Bd 60K is a 57-kDa α-subunit of β-conglucinin. It is a glycoprotein composed of 543 amino acids well known as a major soybean storage protein (Ogawa et al., 2000)**.**

I.V Tree nut allergens

The Food Allergy and Anaphylaxis Network (FAAN) estimates that 0.2% of children and 0.7% of adults are allergic to tree nuts. Tree nuts are divided into 12 major types: hazelnuts, almonds, pistachios, walnuts, pecans, cashew, macadamia nuts, coconut, Brazil nuts, pine nuts, black walnuts, and chestnuts (Teuber et al., 2003). By order of prevalence, allergic reactions to tree nuts in the USA can be ranked as follows: reactions to walnuts (34%), cashew (20%), almonds (15%), pecan (9%), pistachio (7%), and other tree nuts (<5%) (Ahn et al., 2009; Cox et al., 2015). The prevalence varies between regions: in Europe, hazelnut is the tree nut that is most commonly responsible for allergic reactions (McWilliam et al., 2015).

Even though tree nuts are responsible for lethal anaphylactic reactions, characterization of their allergens and epitopes is still incomplete. To date, the major tree nut allergens identified are seed

storage proteins such as vicilins (7S globulins), legumins (11S globulins), and 2S albumins (Cox et al., 2015).

Worldwide, 20 species of walnut trees have been inventoried, the most widely consumed being the English walnut (Cox et al., 2015). In English walnuts (*Juglans regia),* four allergens have been identified: Jug r 1 (2S-albumin), Jug r 2 (a 7S vicilin-like protein), Jug r 3 (lipid transfer proteins), and Jug r 4 (a legumin-like 11S seed storage protein) (Crespo et al., 2006). In black walnuts, which are far less consumed than English walnuts, two allergenic proteins (*Juglans nigra*) have been identified: Jug n 1 and Jug n 2 (Cox et al., 2015).

Cashew (*Anacardium occidentale)* contains 3 allergens that have been characterized: Ana o 1 (7S vicilin), Ana o 2 (11S globulin), and Ana o 3 (2S albumin). Ana o1 is considered a major allergen, and three immunodominant linear epitopes have been identified (Cox et al., 2015).

To date, fourteen hazelnut allergens (*Corylus avellana*) have been characterized: Cor a 1 to Cor a 14. The major allergen Cor a 1 (PR-10) has 4 isoforms, Cor a 1.01 to 1.04. Cor a 1 isoforms revealed amino acid sequences identities between, 96 and 99%, engendering different antigenic and allergenic properties to the different isoforms (Breiteneder et al., 1993). Four sub-isoforms of Cor 1.04 are known: Cor a 1.0401 to 1.0404 with an amino acid sequence identities among each other between 97 and 99% (Lüttkopf et al., 2002). Cor a 1.04 is a major hazelnut allergen and one immunodominant epitope has been listed [aa 142-153] (Bohle et al., 2005).

Almond (*Prunus dulcis)* is the most consumed tree nut in the world. Currently, five allergens have been found: Pru du 1 (PR-10), Pru du 3 (nsLTP), Pru du 4 (Profilin), Pru du 5, and Pru du 6 (11S globulin). Recognized by 50% of sera from allergic patients, Pru du 6 is classified as a major allergen (Cox et al., 2015).

Pecan nuts (*Carya illinoinensis*) have only two characterized allergens: Car I 1 (2S albumin) and Car I 4 (11S legumin). Car I 4 is the major pecan allergen. Three immunodominant epitopes have been found in the acidic subunits [aa 118-132], [aa 208-219] and [aa 238-249] (Sharma et al., 2011).

In pistachio (*Pistachio vera*), five allergens have been identified: Pis v 1 to v 5. Pis v 1 (7 kDa - 2S albumin) and Pis v 2 (32 kDa - 11S globulin) are considered major allergens, with 68% and 50% IgE binding, respectively (Ahn et al., 2009; Cox et al., 2015).

Brazil nuts (*Bertholletia excela*) have two allergens: Ber e 1 and Ber e 2 (Cox et al., 2015).

To complicate things further, patients that are allergic to a particular allergen can also react to other allergens because of high homology between proteins. This feature is referred to as cross-reactivity.

I.VI Cross-reactivity

Cross-reactivity is the recognition of different antigens by the immune system or by an immuno-enzymatic method, due to a high degree of similarity or sequence homology between allergenic proteins (Frank, 2002).

Three-quarters of the birch-pollen-allergic population will have an allergic reaction after consumption of certain raw fruits (apple and kiwi), nuts (hazelnut, walnut), and vegetables (celery, carrot), as these contain proteins highly homologous to the major allergen of birch pollen (*Betula verrucosa*) Bet v 1 (Bohle et al., 2005; Asero et al., 2007). For example, the major allergen of hazelnut (Cor a 1.04) shares high homology (79%) with bet v1 (birch pollen) and (71%) with Cor a1 (hazel pollen) (Bohle et al., 2005).

The major allergens in peanut, i.e. 2S albumins, vicilin, legumins, and profilins, share homology with many tree nuts (Sharma et al., 2011; Cox et al., 2015). For example, Ara h 2 has epitopes similar to those recognized by IgE in almonds and Brazil nuts (De Leon et al., 2007). A study performed on 142 peanut-allergic patients showed 50% positive skin tests for almonds, 40% for cashews, 30% for pistachios, 26% for Brazil nuts, and 21% for hazelnuts (Crespo et al., 2006). The Car I 4 shares 65% to 97% homology with several leguminins, such as Jug r 4, Cor a 9, and Ana o 2 (Sharma et al., 2011).

In addition, the high homology between walnuts and pecans (both of the *Juglandaceae* family) and between cashew and pistachio (both of the *Anacardiaceae* family) also leads to major cross-reactivity between these tree nuts (Cox et al., 2015). As a consequence, the high homology between tree nuts makes it hard to distinguish the origins of these allergens by immunoenzymatic methods such as Enzyme-linked immunosorbent assay ELISA (De Leon et al., 2007; Willison et al., 2008; Noorbakhsh et al., 2011).

Section I: Adverse food reactions: mechanisms, diagnosis, and treatments

A food can trigger an allergic reaction (immune-mediated hypersensitivity), food poisoning, or food intolerance (**Figure 2**) (Ozdemir et al., 2009). According to several studies, based on self-diagnosis, between 5 and 30% of the children claim to suffer from food allergy or intolerance in Europe (McBride et al., 2012; Nwaru et al., 2014). One should note, however, that the prevalence of adverse reactions to food, as supported by the results of controlled food challenge trials (consumption of the target allergen under clinical supervision), is six times lower than the self-reported prevalence (Muraro et al., 2014).

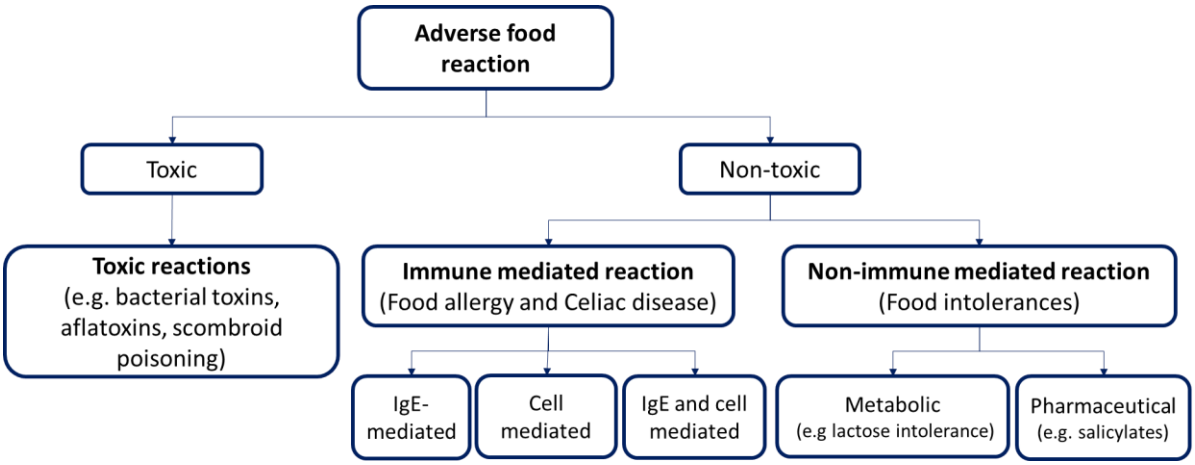


Figure 2: *Adverse reactions to food products can be divided into 2 categories: toxic (toxic reactions) and non-toxic (immune-mediated and non-immune-mediated reactions) (modified from* (Sampson et al., 2014)).

It is important to make the distinction between allergy/intolerance and toxic reactions. The latter cannot be considered a food intolerance, as it is a circumscribed event due to the release of toxic substances into the organism and affecting all people who ate the product (Ozdemir et al., 2009).

Several examples of food intolerance (e.g. to lactose, to sugars) and the reasons for their occurrence are briefly presented below, so as to better explain the difference between an intolerance and an allergy. This is followed by a detailed presentation of food allergy in relation to the mechanisms of its development, with an up-to-date description of the currently available diagnostic methods and treatments.

II Food intolerance

Food intolerance is defined as an adverse reaction to food that does not involve the immune system. It can be due to a metabolic food disorder (e.g. lactose intolerance caused by reduced intestinal lactase activity) or a pharmacological reaction (e.g. tyramine: a trace amine derived from tyrosine, found in meat, cheese, red wines…, known to act as a catecholamine-releasing agent) (Ortolani et al., 2006; Maintz et al., 2007; Leung et al., 2014). So far, no biological markers for confirming food intolerance are known (Wilson, Kate, Hill, 2014).

II.I Metabolic food disorders

Carbohydrate malabsorption can be caused by a deficiency of digestive enzymes (e.g. lactose or sorbitol intolerance) or by altered uptake of certain mono- or di-saccharides (fructose, glucose, lactose…) (Cuatrecasas et al., 1965; Ozdemir et al., 2009; Zopf et al., 2009).

II.I.1 Digestive enzyme deficiencies

β-galactosidase (lactase), a digestive enzyme, is responsible for the hydrolysis of lactose, a disaccharide, to the monosaccharides glucose and galactose in the small intestine. Some 70% of adults have low lactase activity (lactase deficiency) resulting in poor lactose digestion (Lomer et al., 2007). Generally, however, symptoms do not occur unless lactase activity is below 50% of its normal level (Lomer, 2015). The lack of lactose absorption by the small intestine leads to the presence of lactose in the colon, and this generates symptoms. Lactose fermentation by prokaryotes in the colon can cause abdominal pain, flatulence, diarrhea and bloating (Lomer et al., 2007). In addition, lactose intolerance depends on the individual and on the ingested quantity, as many individuals displaying lactase deficiency can consume small amounts of milk (e.g. 12 – 15 g lactose/day, corresponding to 20–30 ml skimmed cow's milk). To avoid uncomfortable symptoms, lactose must be excluded from the diet (Ozdemir et al., 2009; Lomer, 2015).

II.I.2 Carbohydrate transport deficiencies

Carbohydrates (mono-, di-, oligo-, and polysaccharides) are the major contributors to our current diet (e.g. breads, pasta, cakes, chocolates, candies…). Their early introduction into infant formulae induces premature digestion of polysaccharides and an increase in the prevalence of metabolic food disorders (Holzel, 1967).

Fructose is a monosaccharide naturally present in fruits, vegetables, and honey. The uptake of fructose by the epithelium of the small intestine is ensured by two glucose transport protein

isoforms (GLUT 5 and GLUT 2) expressed in several tissues (small intestine, kidney, brain…) (Putkonen et al., 2013; Wilder-Smith et al., 2014). The mechanisms responsible for fructose malabsorption are controversial and not fully understood, but could involve a reduction of GLUT 5 expression, a gene regulated by glucose and thyroid hormone (Matosin-Matekalo et al., 1999; Wilder-Smith et al., 2014). One should note, furthermore, that a clinical study performed on 11 patients with fructose intolerance and 15 controls revealed no deficiency in GLUT5 or GLUT2 expression in the fructose-intolerant patients. More investigations are thus needed to determine the real cause of fructose intolerance (Wilder-Smith et al., 2014; Ebert et al., 2016). Non-absorption or poor absorption of fructose in the small intestine leads to its fermentation by bacteria, yielding short-chain fatty acids and gases (hydrogen, carbon dioxide, methane). This results in gastrointestinal symptoms such as diarrhea, excess of gas… (Gibson et al., 2006; Shepherd et al., 2006).

II.II Pharmaceutical reactions

Food intolerance can also be induced by food chemicals such as salicylates and amines (Lessof, 1985; Ozdemir et al., 2009). Salicylates are chemicals naturally present in some food products such as tomato-based sauces, fruit and fruit juice, tea, wine, spices… (Ozdemir et al., 2009; Duthie et al., 2011). In food-intolerant populations, mast cells are activated by salicylates to overproduce leukotrienes (inflammatory mediators) such as LTC4. This leads to smooth muscle contractions, an inflammatory reaction (angioedema), respiratory problems (bronchial asthma), and gastrointestinal problems (abdominal pain, diarrhea, swelling) (Raithel et al., 2005; Togo et al., 2009; Lomer, 2015). Histamine and tyramine are biogenic amines present in numerous food products (tuna, bananas, wine, tomatoes…) and eliminated by amine oxidases (diaminoxidase for histamine) in the healthy population. However, low amine oxidase activity leads to a risk of amine accumulation and toxicity, as an increased amine level causes smooth muscle contractions, headache, and cutaneous, gastrointestinal (diarrhea), and cardiac (arrhythmia) complications (Maintz et al., 2007; Zopf et al., 2009).

III Food allergy

III.I The immune response: a rapid overview of the main actors

To defend the organism, immune responses are established. These include innate immunity (an immediate and non-specific response) and adaptive immunity (a slow and specific response) (**Figure 3**). A brief overview of the cells involved in the defense of the organism, the basic concepts, not

directly related to food allergies, will be presented to understand better the mechanism of food allergy.

The innate immune system does not respond specifically, unlike the adaptive immune system which has antigen-specific lymphocytes (T and B cells) to combat infections (Janeway et al., 2009).

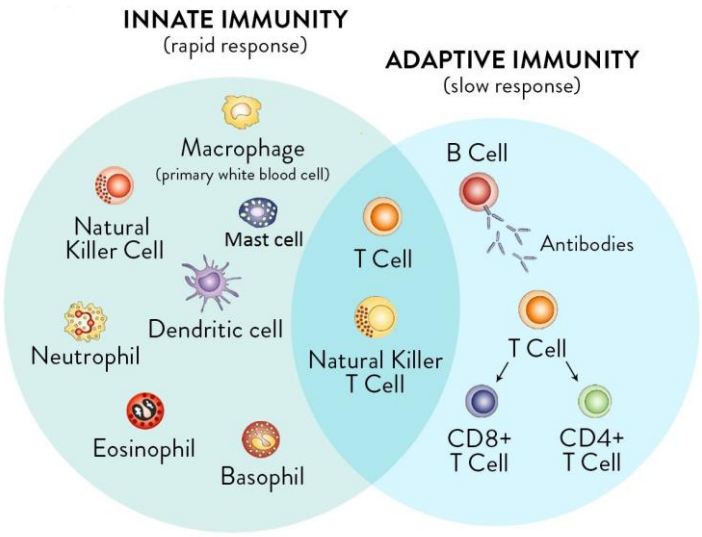


Figure 3: *The innate and adaptive immune systems. Cells involved in innate immunity include granulocytes (basophils, eosinophils, and neutrophils), mast cells, macrophages, dendritic cells, T lymphocytes, and natural killer cells. Participants in adaptive immunity include antibodies, B cells, and CD4⁺ and CD8⁺ T lymphocytes (modified from* (Dranoff, 2004)*).*

III.I.1 The innate immune response

First protection against pathogens is provided by the body surface (skin) and the mucosal epithelia (of the respiratory, gastrointestinal, urinary, and reproductive tracts). Their cumulated surface reaches 200-300 m² in adulthood. Antimicrobial peptides produced and secreted by the epithelia and by circulating immune cells (neutrophils and monocytes) protect the host from infections (Dommett et al., 2005).

When a pathogen crosses the epithelial barrier, pathogen-associated molecular patterns (PAMPs) are detected by a limited number of pathogen recognition receptors (PRRs) (Medzhitov et al., 2000; Akira et al., 2006). PRRs, expressed by innate immune cells such as dendritic cells, macrophages, and neutrophils, belong to several families and include the Toll-like receptors (TLRs), the retinoic acid–inducible gene RIG-I-like receptors (RLRs), nucleotide binding oligomerization domain NOD-like receptors (NLRs), Deoxyribonucleic acid (DNA) receptors (cytosolic sensors for DNA) and C-type

lectin receptors (BOX 3) (Hoebe et al., 2004; Hammad et al., 2008; Kumar et al., 2011). Innate cells can distinguish self- from non-self-molecules thanks to the molecular signatures, PAMPs of pathogens, such as the presence of peptidoglycans and liposaccharides on the surfaces of bacteria but not on the surface of eukaryotic hosts, with an exception in the case of viral pathogens produced in the host cells (Medzhitov, 2002; Hoebe et al., 2004).

Recognition of PAMPs of extracellular or intracellular pathogens by PRRs triggers a quick, efficient immune response characterized by release of pro-inflammatory cytokines, chemokines, and type I interferon (Medzhitov et al., 2000; Saraiva et al., 2010; Kumar et al., 2011). Cytokines and chemokines are soluble immune mediators, released by several cell types as communication signals (e.g. stimulating or inhibiting the proliferation, differentiation, and/or activity of immune cells). The main functions of the innate cells involved in fighting food allergens are briefly presented below.

Macrophages and **neutrophils**, are recruited to the infected site by the activated endothelium and by chemokines. For example, macrophages secrete interleukin IL-8, which enhances the recruitment of neutrophils, basophils, and T cells to the site of infection. They also very efficiently phagocytose pathogens, a process involving several receptors (mannose receptor, fragment crystallizable (Fc) receptors) (Janeway et al., 2009). The mannose receptor expressed in the plasma membrane of macrophages and immature dendritic cells recognizes mannose and fucose present at the surface of pathogens and triggers phagocytosis (Engering et al., 1997; Aderem et al., 1999). Phagocytosis is defined as the ingestion and destruction of a pathogen by phagocytes, with the help of a variety of toxic products (the superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), nitric oxide ($NO^{\bullet}$)…) that contribute to killing the engulfed microorganism (Janeway et al., 2009) (**Figure 4**).

Natural killer (NK) cells kill virus-infected cells by releasing perforins and granzymes. These factors perforate the membrane of the infected cell, triggering apoptosis (a mode of cell death). The killing activity of NK cells is monitored by the recognition of cells deficient in major histocompatibility complex (MHC) cell surface expression, highly expressed at the surfaces of most vertebrate cells (Alberts et al., 2002; Campbell et al., 2013). The release by NK cells of cytokines such as interferon-γ (IFN-γ) allows macrophage activation and promotes a subsequent adaptive response (Abbas et al., 2016).

Dendritic cells (DC), like macrophages, are antigen-presenting cells (APCs). Dendritic cells present to naive T lymphocytes (cells that have never met the antigen) an antigenic peptide bound to a MHC protein, in order to establish an immune response (Banchereau et al., 2000) (**Figure 5**). They recognize pathogens via several receptors, such as TLRs, NLRs, and BOX 3 (Hammad et al., 2008). After PAMP recognition by dendritic cell receptors, the molecules are hydrolyzed via the

endolysosomal pathway by lysosomal enzymes (Figura et al., 1986; Bieber et al., 2002; Villadangos et al., 2007). The resulting protein-derived peptides are loaded onto an MHC class II protein for presentation and then activate the adaptive immune response (Bieber et al., 2002).

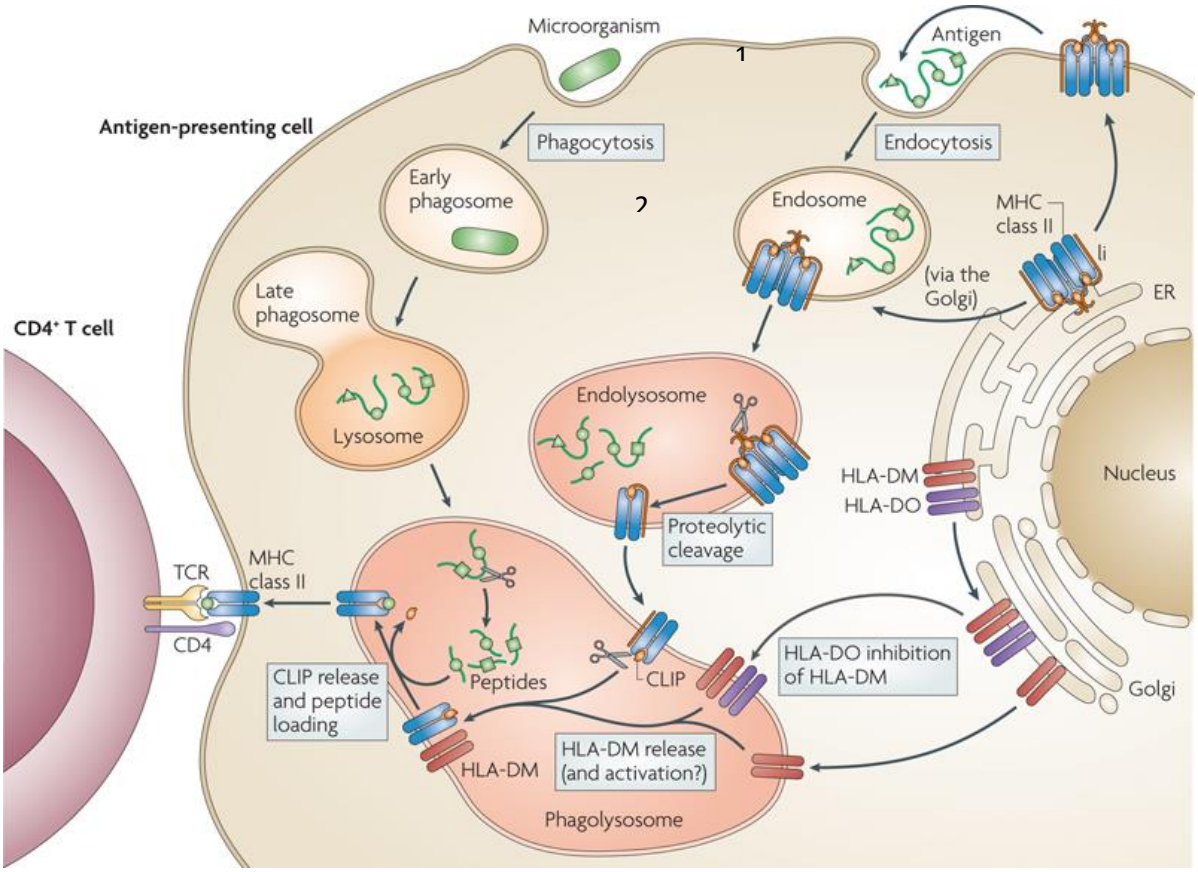


Figure 4: *Mechanisms of phagocytosis and endocytosis in response to infectious agents: (1) chemotaxis (attraction of phagocytes to microbes or a site of infection) and adherence; (2) ingestion by phagocytes; (3) digestion or killing and (4) exocytosis (elimination) or loading of an antigenic peptide onto the major histocompatibility complex (MHC) class II to initiate the adaptive immune response. MHC class II is assembled in the endoplasmic reticulum and is a heterodimer comprising an α and a β chain (from* (Harding et al., 2010)*).*

Mast cells and **basophils** are tissue-based inflammatory cells responding to signals from innate and adaptive immunity effectors by immediate or delayed release of inflammatory mediators (e.g. histamine, serine proteases (tryptase and chymase), carboxypeptidase A, proteoglycans). In allergic population, IgEs are bounded to the high-affinity immunoglobulin ε fragment crystallizable region receptor I (FcεRI) expressed on mast cells and basophils. After recognition of an antigen by a specific IgE, FcεRI aggregation and the release of inflammatory mediators (Galli et al., 2005; Caughey, 2007; Amin, 2012).

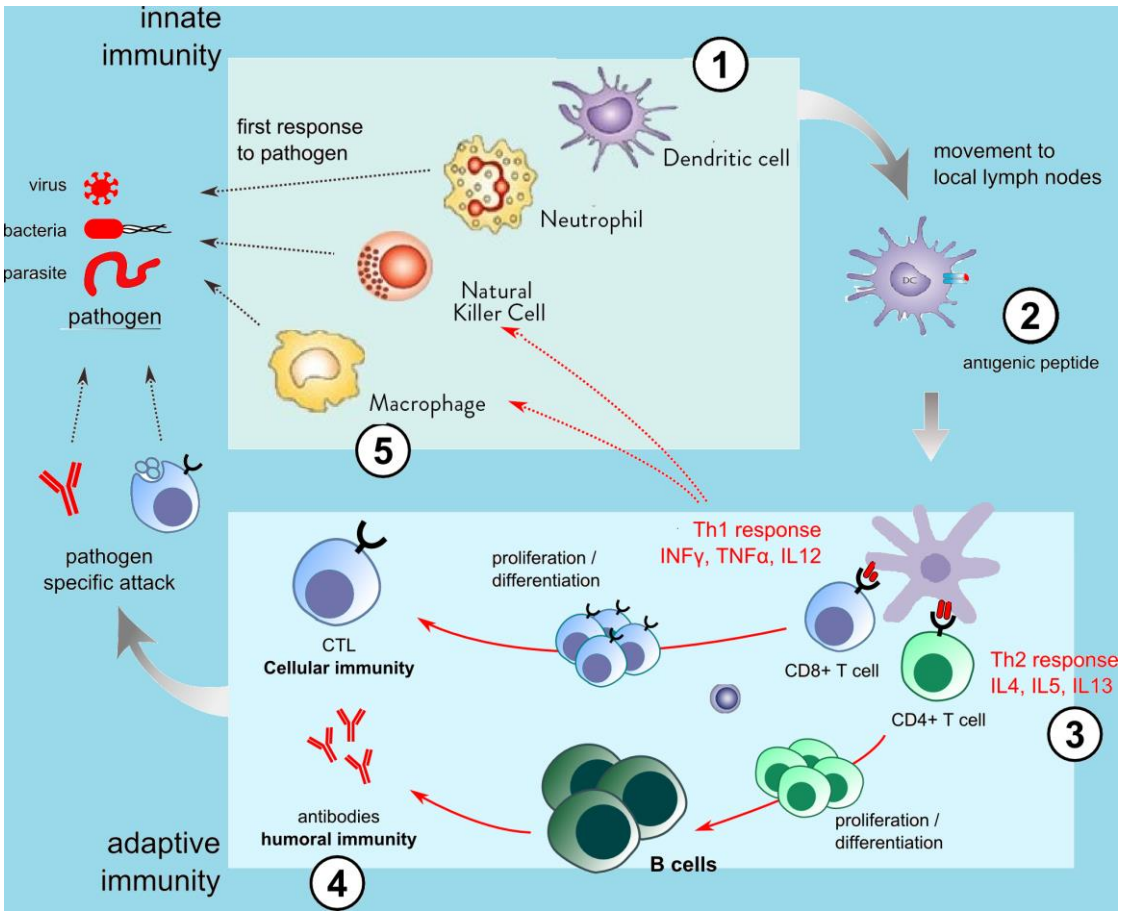


Figure 5: *Innate and immune responses to pathogens. Antigen-presenting cells (dendritic cells and macrophages) present MHC-class-II-borne antigenic peptides to naive T cells. Recognition of an antigenic peptide by naive T cells enhances the differentiation and proliferation of T helper 2 (T_H2) cells. Naive B cells are activated by T_H2 cells or through recognition of the antigen by B cell receptors and enhance the humoral response, producing specific IgE/IgG antibodies (modified from* ('Vaccine adjuvants'.)*).*

Eosinophils release their content of cytotoxic mediators. These include four cationic proteins (eosinophil peroxidase (EPO), major basic protein (MBP), eosinophil cationic protein (ECP) and eosinophil-derived neurotoxin (EDN)), various pro-inflammatory cytokines (IL-2, IL-4, IL-5, IL-12, IL-13, IL-16, IL-18), chemokines (Hogan et al., 2008). They are involved in tissue damage and allergic responses such as asthma (Shamri et al., 2011). Eosinophils are also viewed as immunoregulatory cells as they synthetize anti-inflammatory mediators (IL-10 and transforming growth factor (TGF)) and as potential effectors playing an important role in both innate and adaptive immune responses (Legrand et al., 2008).

III.I.2 The adaptive immune response

The adaptive immune response is specific and provides long-lasting immune protection. The adaptive immune response requires B cells, which mediate a humoral response leading to antibody production, and T cells, which mediate cellular responses (Leung et al., 2010; Abbas et al., 2016). Recognition of an antigen by a T-cell receptor (TCR) enhances the proliferation of T lymphocytes and their differentiation into one of the three classes of T cells. Cytotoxic $CD8^+$ T cells recognize antigens presented on target cells by the MHC class I. Their function is to kill infected cells. The killing mechanism involves activation by the $CD8^+$ cells of caspases (cysteinyl aspartate proteases), i.e. enzymes that cleave proteins after aspartic acid (Bennett et al., 1998).

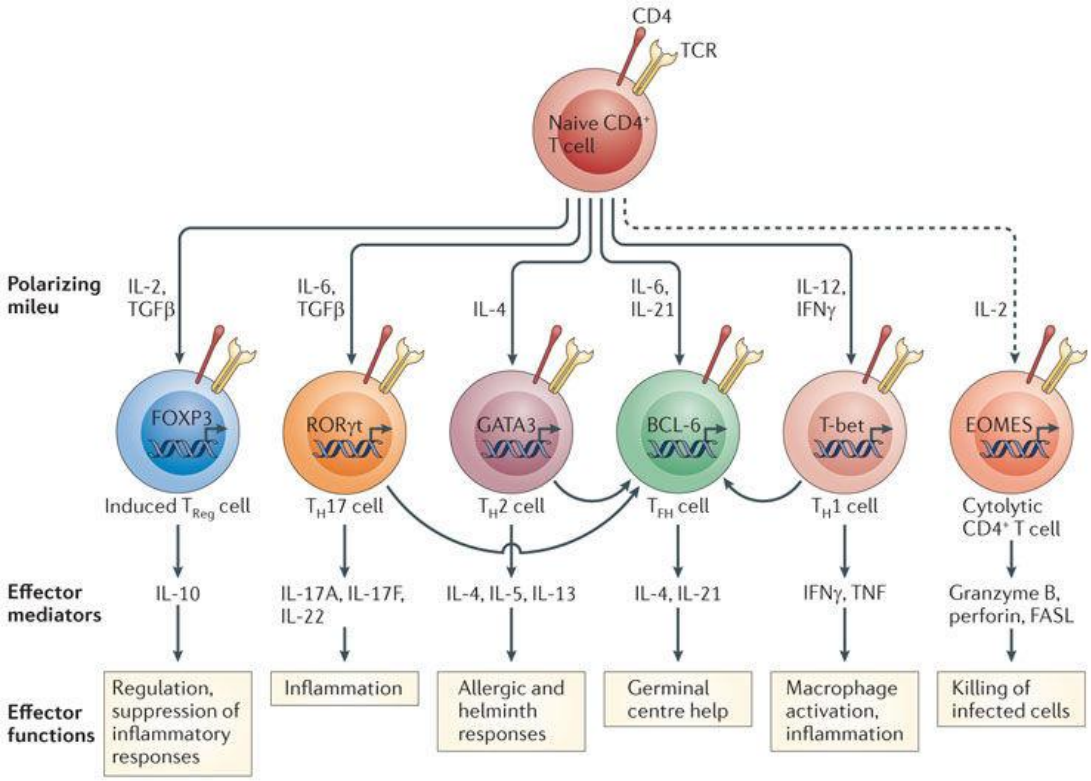


Figure 6*: Differentiation of naive $CD4^+$ T cells, according to the presence of interleukins, interferon-γ or transforming growth factor (TGF), to T_H1 cells participating in cell-mediated immunity (IL-12, IFN-γ), T_H2 cells involved in humoral immunity (IL-4), T_H17 cells involved in cell-mediated inflammation (TGF-β, IL-6), follicular helper cells T_{FH} (IL-6, IL-21) or immunoregulatory T_{reg} cells (IL-2, TGF-β)(from* (Swain et al., 2012)*) .*

The CD4+ T helper cells (T_H) recognize antigens presented on target cells by the MHC class II. They provide essential additional signals that influence the behavior and activity of other cells, such as B-cells (production of antibodies) and macrophages (phagocytosis). Native helper T cells can

differentiate into T_H1, T_H2, T_H17, T_{FH} or T_{reg} cells, depending on the cytokines by which they are stimulated and on the transcription factors activated (**Figure 6**) (Alberts et al., 2002).

Regulatory T cells (T_{reg}) suppress the activity of other lymphocytes and help to control immune responses (homeostasis) (Seddon et al., 2003; Rosado et al., 2015), but to ensure a rapid and effective adaptive response after the second challenge by the target pathogen, memory B and T cells are conserved (Pennock et al., 2013; Abbas et al., 2016) (**Figure 7**).

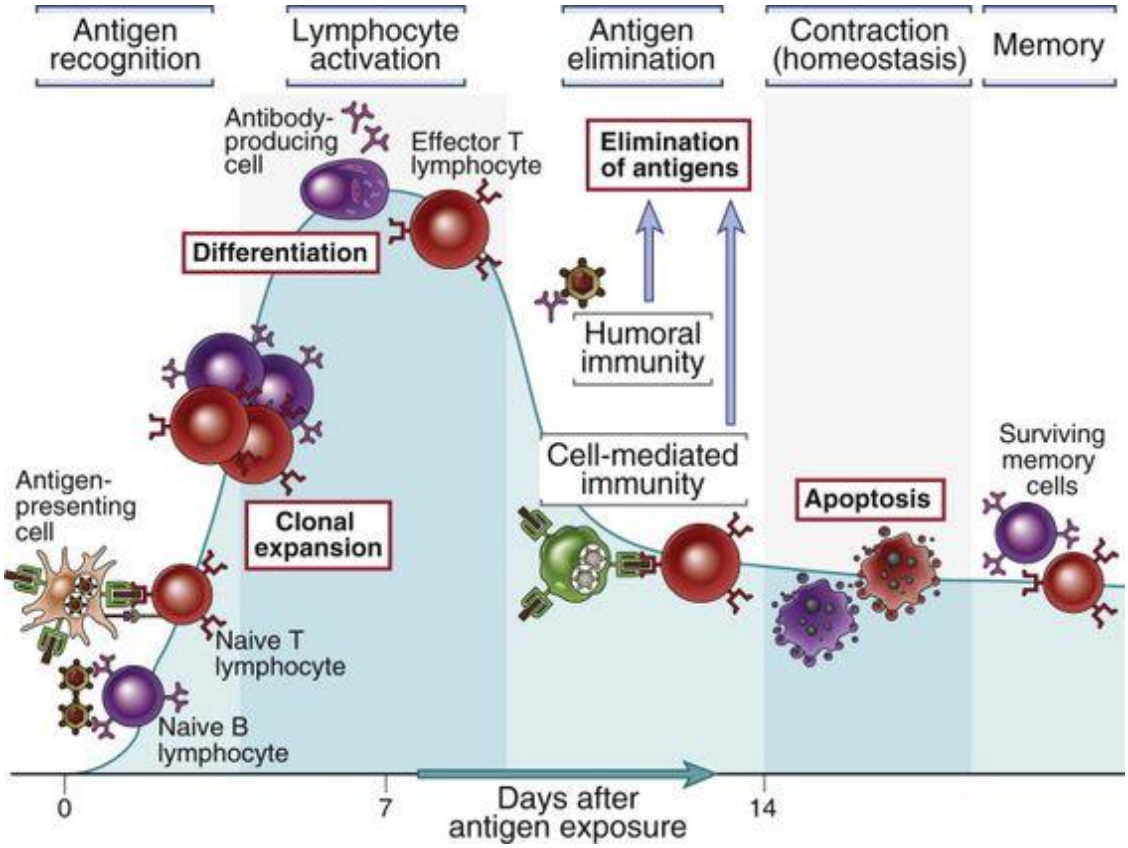


Figure 7*: Phases of an adaptive immune response. Antigen-presenting cells (dendritic cells) trigger the adaptive immune response through recognition of the antigen by naive T and B cells. After clonal expansion, differentiation results in the secretion of antibodies by the plasmocytes and effector T cells. The response declines as antigen-stimulated lymphocytes die by apoptosis (cellular death genetically and biochemically regulated) restoring introduction of homeostasis. The surviving cells are responsible for memory (from* (Abbas et al., 2011)*).*

After the differentiation of naive T cells to T_H2 cells, naive B cells are activated and differentiate to antibody-secreting plasmocytes (Duchosal, 1997). B-cell activation can occur through recognition of the antigen by B cell receptors (IgMs and IgDs on naive B cells) or through helper-T-cell stimulation. In the latter case, a TCR recognizes an antigenic peptide presented by an MHC class II on the B cell, and a second signal is provided by interaction between the CD40L ligand on the T cell and the CD40 receptor on the B cell (McHeyzer-Williams et al., 2012).

III.I.3 Mechanisms of immunotolerance

The lack of immune response to an antigen is called the immune tolerance. The immune tolerance to both self-antigens and non–self-antigens is essential to protect the host against chronic inflammatory diseases and tissue damages (Zeng et al., 2015). Regulatory B- and T-cells (Breg and Treg) with their suppressive cytokines such as IL-10, TGF-β are essential for the induction of immunotolerance (Palomares et al., 2017).

B cells also regulate the immune response through the secretion of cytokines and their surface molecules. Indeed, immunosuppressive cytokines (IL-10, IL-35 and TGF-β) are secreted by regulatory B cells (Breg) and promote the immune tolerance (Van de Veen et al., 2016) (**Figure 8**).

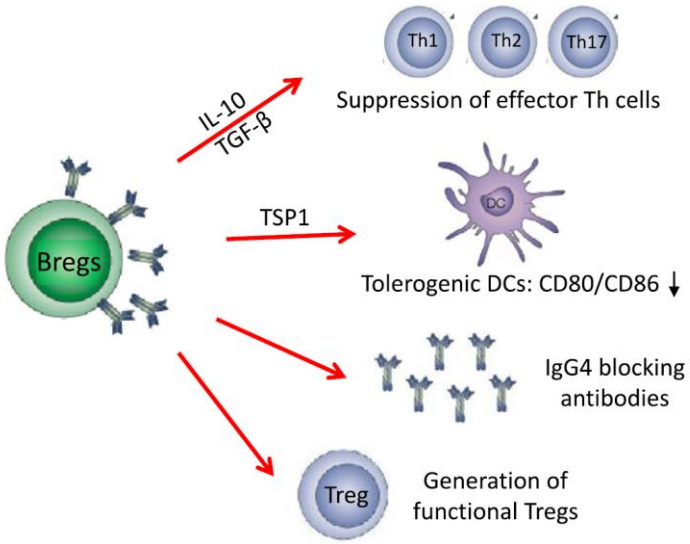


Figure 8: *Role of Breg cells in the allergen tolerance induction and the regulation of inflammation. Breg cells suppress effector T_H cells, induce immune tolerance of DCs, promote the production of blocking IgG4 and functional Tregs (from* (Palomares et al., 2017)*).*

Regulatory T cells (Tregs) are specialized for immune suppression and play a crucial role for the regulation of the central and peripheral immune tolerance. Tregs are produced in the thymus (tTreg) as a functionally mature subpopulation of T cells but can also be derived from naïve T cells in the periphery after antigenic stimulation (pTregs) (Sakaguchi et al., 2008). Peripheral Treg cells are $FOXP3^+$ T cells, IL-10-producing Treg cells (Tregs1), and TGF-β-producing T_H3 cells (Palomares et al., 2017). Treg1 plays an important role in the induction and maintenance of the immune tolerance by an important production of IL-10, an immunosuppressive and anti-inflammatory cytokine (Galli et al., 2008). Indeed, Treg1 inhibits effector T cells and may kill APC using perforin and granzymes (Sakaguchi et al., 2008). The mechanism of T cell tolerance includes major mechanism of central tolerance such as the clonal deletion and major mechanism of peripheral tolerance as the anergy

(tolerance mechanism in which the lymphocyte is functionally inactivated (Schwartz, 2003)), the exhaustion (state of T cell dysfunction defined by poor effector function (Wherry, 2011)), and suppression (regulatory T cells suppress T-cell activation (Ling et al., 2004)) (Zeng et al., 2016) (**Figure 9**).

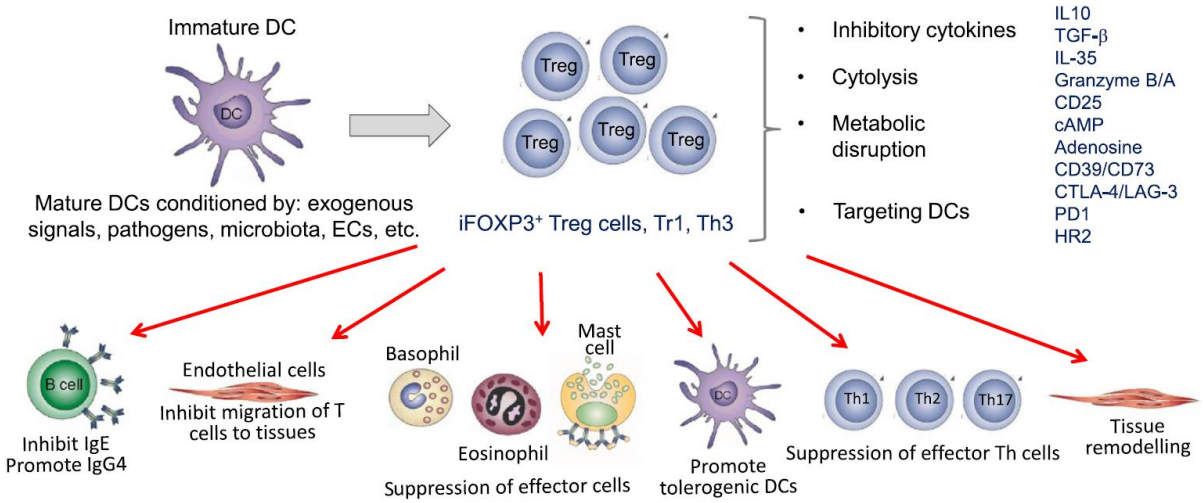


Figure 9: *Treg cells suppress allergic reactions by the inhibition of inflammatory events. There are four main groups of suppressive mechanisms: suppressive cytokines (IL-10, TGF-β, and IL-35), cytolysis (cell bursts and releases its contents) (granzymes A and B), metabolic disruption mechanisms (CD25, cAMP, adenosine receptor 2, histamine receptor 2, CD39, and CD73), suppression of DC activation by membrane-bound molecules (CTLA-4, PD-1) (from* (Palomares et al., 2017)*).*

The immune and adaptive immune response participates in food allergy reactions. The breakdown of tolerance of food allergens is still unclear even if several hypotheses have been suggested such as an increased intestinal permeability that could be a potential cause for the breakdown in tolerance, an alternative routes of food allergen exposure, such as through the skin or the respiratory tract, or a failure in Treg activity (Wang et al., 2011). However, genetic and environment (pollution, dietary habit…) have also a strong influence on the development of food allergy (Mübeccel et al., 2009). Below, after a description of the four types of hypersensitivity reaction (allergy), the different phases and mechanisms involved in the development of food allergy are described in detail.

III.II Classes of hypersensitivity reactions

Allergy or hypersensitivity is caused by disorders of the immune system that trigger an adaptive immune response. The mechanisms of allergy development represent four major strategies that

the body uses to combat infectious agents which involve different cell types depending on the type of pathogen encountered (food, antibiotic, pollen…) (Palm et al., 2012).

In the early 1960s, Coombs and Gell classified hypersensitivity reactions into four types I-IV (**Table 1**) (Coombs, 1963): type I (IgE-mediated), type II (cytotoxic or IgG/IgM-mediated for cell or matrix antigens), and type III (IgG/IgM immune-complex-mediated for soluble antigens) are humoral (B-cell-dependent) immune responses, while type IV hypersensitivity is delayed and is a reaction mediated by T cells (T, T_H1, T_H2, or CTL).

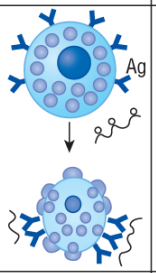
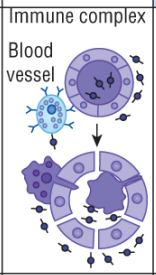
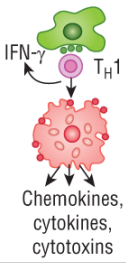
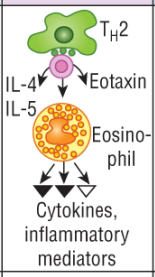
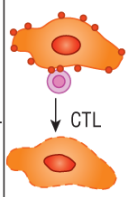
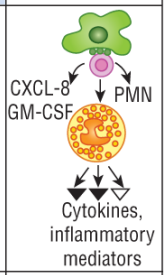
	Type I	Type II	Type III	Type IVa	Type IVb	Type IVc	Type IVd
Immune reactant	IgE	IgG	IgG	IFNγ, TNFα (T_H1 cells)	IL-5,IL-4/IL-13 (T_H2 cells)	Perforin/ granzyme B (CTL)	CXCL-8, IL-17 GM-CSF (T-cells)
Antigen	Soluble antigen	Cell-or matrix-associated antigen	Soluble antigen	Antigen presented by cells or direct T-cell stimulation	Antigen presented by cells or direct T-cell stimulation	Cell-associated antigen or direct T-cell stimulation	Soluble antigen presented by cells or direct T-cell stimulation
Effector	Mast cell activation	FcR^+ cells (phagocytes, NK cells)	FcR^+ cells complement	Macrophage activation	Eosinophils	T-cells	Neutrophils
							
Example of hypersen-sitivity reaction	Allergic rhinitis, asthma, systemic anaphylaxis	Hemolytic anemia, thrombocytopenia (e.g., penicillin)	Serum sickness, Arthus reaction	Tuberculin reaction, contact dermatitis (with IVc)	Chronic asthma, chronic allergic rhinitis Maculopapular exanthema with eosinophilia	Contact dermatitis Maculopapular and bullous exanthema hepatitis	AGEP Behcet's disease

Table 1: *Revised Coombs and Gell classification of hypersensitivity. Hypersensitivities are classified as either antibody-mediated (Types I-III) or T cell/cytokine-dependent (Type IV). AGEP: acute generalized exanthematous pustulosis; PMN: polymorphonuclear neutrophil; CTL: cytotoxic T lymphocyte; GM-CSF: granulocyte-macrophage colony-stimulating factor; CXCL-8: CXC-chemokine ligand 8 (from* (DiPiro et al., 2014)*).*

Food allergies are mostly IgE-mediated reactions (immediate hypersensitivity type I) resulting in mast cell activation, but they can also be IgE-independent (hypersensitivity type IV) (examples: celiac disease and eosinophilic esophagitis) (Sampson, 2004; Valenta et al., 2015).

III.III IgE-mediated food allergy

Type I food allergy is an IgE-mediated reaction characterized by binding of IgE to the high-affinity immunoglobulin ε Fc region receptor I (FcεRI) expressed on mast cells, basophils, and eosinophils. A food allergy involves two phases: (1) a sensitization phase and (2) an activation phase (Coico et al., 2015).

III.III.1 Sensitization phase

In susceptible (atopic) individuals, the first exposure to an allergen (antigen) initiates the sensitization phase, defined by production of specific IgEs without the development of allergic symptoms. Sensitization depends on entry of the allergen through the gastrointestinal tract, the respiratory tract, or through skin contact (Han et al., 2012; Valenta et al., 2015). Food allergens are classified in two classes: class I are water-soluble glycoproteins which are stable to treatment with heat, acid or proteases (peanut, milk, egg…) and class II containing allergens from the plant derived system which are heat-labile and difficult to isolate (apple, celery, cherry…) (Breiteneder et al., 2004 a; Sampson, 2004).

In the present research project, we have focused on the target allergens in milk, egg, soy, peanut, and tree nuts, all of which are considered class 1 food allergens.

- **T_H2 cell activation**

In response to the first exposure to an allergen, dendritic cells located in the epithelia recognize and internalize the antigen via receptors such as C-type lectin receptors (mannose receptors), scavenger receptors, and Toll-like receptors (TLRs). The mannose receptor is a multifunctional endocytic receptor. It includes a cysteine-rich domain recognizing sulfated sugars and a C-type lectin-like carbohydrate domain recognizing mannose (Sallusto et al., 1995; Royer et al., 2010).

The protein is internalized and degraded to peptides via the endocytic pathway. Allergenic peptides are then exposed at the surface of APCs (in association with MHC class II) and presented to the appropriate complementary T-cell receptor (TCR) on a $CD4^+$ T cell (Sallusto et al., 1995). This receptor is expressed by naive T cells (T cells never having met the antigen) located in lymphoid tissues (Sathe et al., 2016) (**Figure 10**).

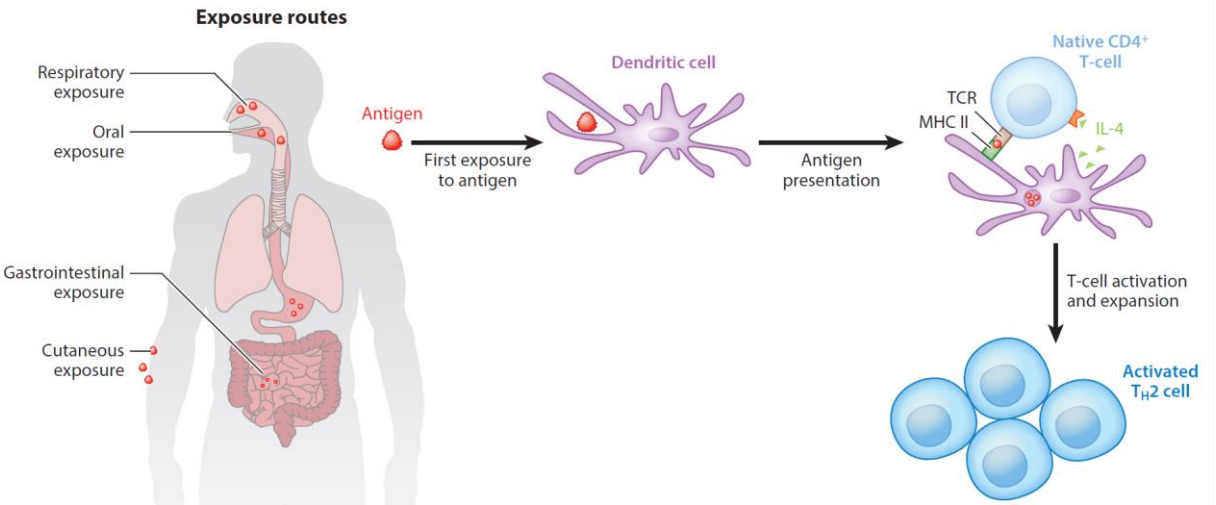


Figure 10: *During the sensitization phase, the allergenic peptide bound to the major histocompatibility complex class II (MHC II) is presented by dendritic cells to T cells. Naive CD4⁺ cells recognize the epitope via the T-cell receptor (TCR). This results in activation of the cells and synthesis and secretion of cytokines (IL-4). The cytokines then polarize, activate, and stimulate proliferation of T_H2 cells. Abbreviations: IL: interleukin; MHC II: major histocompatibility complex class II; TCR: T-cell receptor; T_H2 cell: type-2 helper T cell (from* (Sathe et al., 2016)*).*

Recognition of the antigenic peptide by the T-cell receptor is possible thanks to the particular structure and topology of T-cell receptors. These receptors consist of 2 polypeptide chains (α and β), each defined by a constant (C) and a variable (V) region. Three hypervariable regions allow recognition of the peptide presented by the antigen-presenting cell (**Figure 11**). The MHC class II molecule, involved in type I food allergy, is a transmembrane glycoprotein containing a peptide-binding groove for a peptide composed of 12 – 17 amino acids (Coico et al., 2015).

The function of the MHC molecules expressed on APCs is to present peptides derived from the antigen. MHC class I interacts with the receptor of a $CD8^+$ T cell and stimulates it to kill the infected host cells. MHC class II interacts with the receptor of a $CD4^+$ T cell and triggers activation and cytokine production (interleukins: IL-4, IL-5, IL-9 and IL-13). In response to stimulation by IL-4, the native $CD4^+$ T cells are then polarized, activated, and expanded into type-2 helper T cells (T_H2 cells).

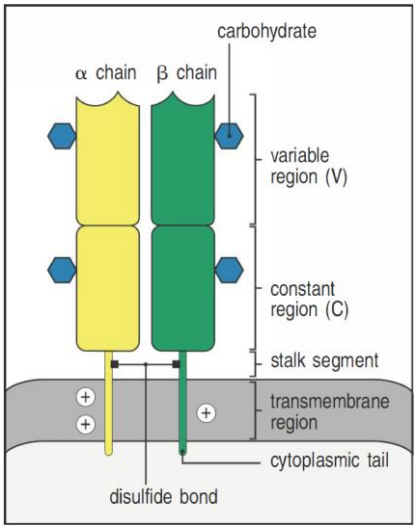


***Figure 11**: Structure of a heterodimeric T-cell receptor (TCR) containing one variable (V) and one constant (C) region on each (α or β) transmembrane glycoprotein chain in order to identify the peptide fragment presented by the MHC class II on the antigen-presenting cell. (from* (Janeway et al., 2009)*).*

- **Initialization of the humoral response**

There are five different classes of immunoglobulins: IgA, IgD, IgE, IgM and IgG associated with the five different heavy chains α, δ, ε, μ and, γ respectively (Wang et al., 2007). Naive B-cells express only IgM and IgD immunoglobulins as receptors. Recognition of an antigen by the IgM and IgD receptors on the naive B cells, followed by activation by T_H2 cells, initiates the humoral response (production of specific IgE antibodies) and the production of cytokines (McSherry et al., 2008). The synthesis and release of cytokines such as IL-4 and IL-13 and the interaction between the CD40 expressed by naive B cells and its ligand on a T_H2 cell allows class switching, i.e. a switch from the production of IgM or IgD (naive B cells) to the production of IgE antibodies with ε heavy chains (Abbas et al., 2016). The class switch by the production of IgE instead of IgM or IgD are due to a change of heavy chains.

- **IgE binding to granulocytes**

Activated B cells (cells secreted IgEs) and T_H2 cells trigger clonal expansion of T_H2 and B cells. IgEs secreted by activated B cells bind to high-affinity IgE receptors (FcεRI) on granulocytes: mast cells in tissues and basophils in the blood (Janeway et al., 2009) (**Figure 12**).

The sensitization phase allows migration of activated T and B cells into several target organs (gastrointestinal tract, respiratory tract, skin, and central nervous system) (Aderbal et al., 2003).

The second exposure to the allergen will induce a rapid immunological response and an allergic reaction (Valenta et al., 2015).

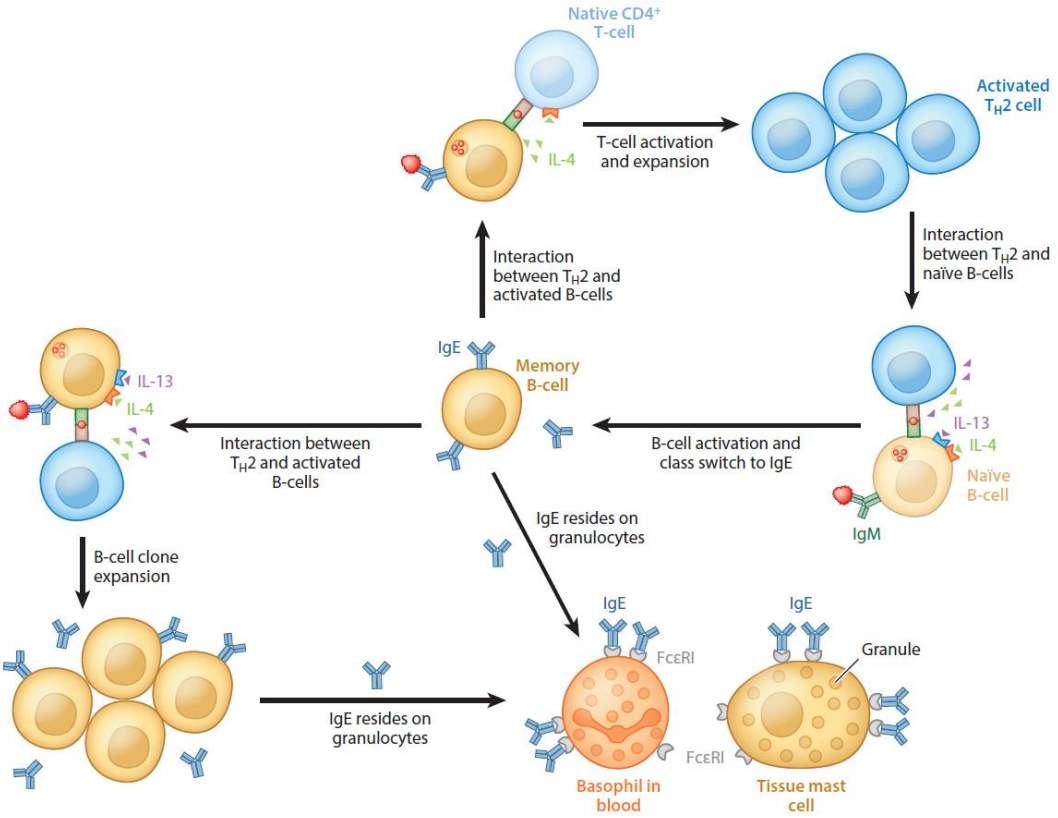


Figure 12: *Interaction between T_H2 cells and naive B cells induces B cell class switching, resulting in production of IgE. IgE antibodies then bind to granulocytes (mast cells and basophils) via the high-affinity receptor FcεRI. Abbreviations: CD4+: cluster of differentiation 4; FcεRI: high-affinity immunoglobulin ε Fc region receptor I; Ig: immunoglobulin; IL: interleukin; T_H2 cell: T helper 2 cell (from* (Sathe et al., 2016)*).*

III.III.2 Activation phase

During the second exposure to the allergen, for the activation phase to occur, at least two IgEs bound to FcεRI receptors on a mast cell or basophil must recognize and bind the antigen. An allergic reaction is initiated by aggregation of the *FcεRI* receptors (this is called clustering), which induces degranulation of mast cells/basophils containing mediators responsible for the immediate and late-phase reactions (Daëron et al., 1995; Valenta et al., 2015; Abbas et al., 2016). During the degranulation step, some 40 mediators are secreted/released: mainly preformed mediators (histamine, tryptase, chymase and heparin), lipid mediators (prostaglandin D_2 (PGD_2), leukotriene C_4, leukotriene D_4 and leukotriene E_4 (LTC_4, D_4 and E_4, respectively) and inflammatory cytokines (IL-3, IL-4, IL-5, IL-8, IL-9, tumor-necrosis factor-alpha (TNF-α) and granulocyte macrophage – colony

stimulating factor (GM-CSF) and chemokines (Jutel et al., 2011; Johnston et al., 2014; Coico et al., 2015) (**Figure 13**).

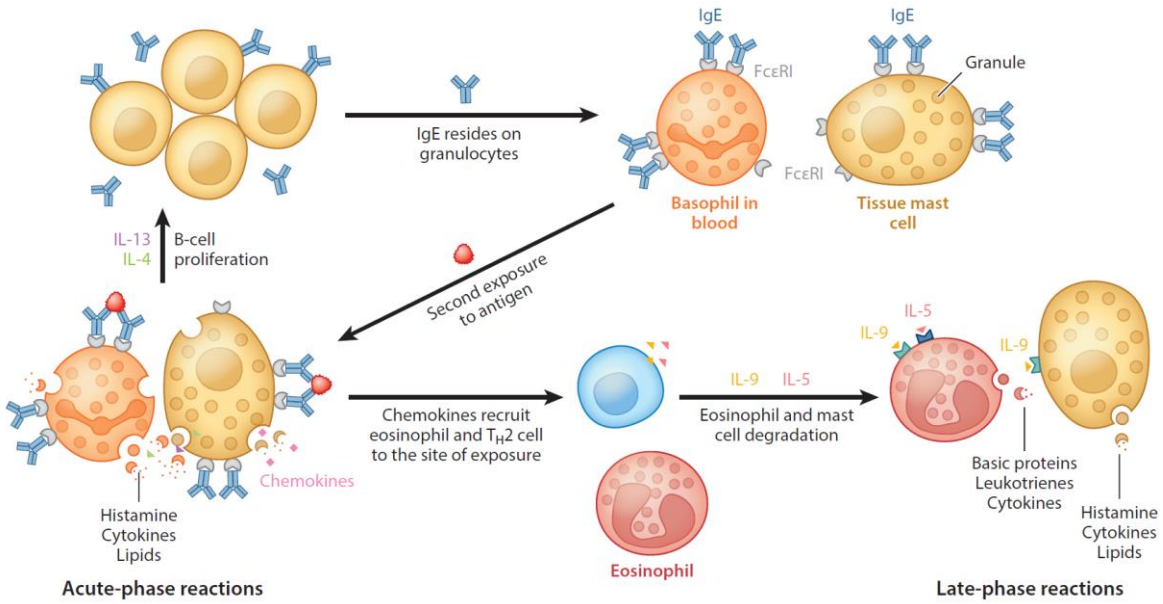


Figure 13: *The activation phase (in response to a second exposure to the allergen) activates mast cells and basophils which release mediators and cause acute- and late-phase allergic reactions. Released mediators play a crucial role in cell recruitment (from* (Sathe et al., 2016)*).*

The late-phase reactions occur several hours after the immediate reaction and involve inflammatory cells (Johnston et al., 2014). The chemokines synthesized by mast cells recruit T_H2 cells and eosinophils to the reaction area. The release of mediators (IL-5 and IL-9) by T_H2 cells activates mast cells and eosinophils and induces late-phase reactions through the release of several mediators (**Figure 13**) (Sathe et al., 2016). Eosinophils secrete proteases causing tissue damage and inflammation of the gastrointestinal tract, while T_H2 cells exacerbate the reaction by producing cytokines (Carrard et al., 2015).

The roles and effects of the main mediators involved in the allergic reactions are briefly described below.

Histamine provokes blood vessel dilation and an increase in vascular permeability, causing edema and a drop in blood pressure. It also stimulates smooth muscle contraction around the bronchi in the lung, causing asphyxia (Cianferoni et al., 2012; Coico et al., 2015; Abbas et al., 2016).

The prevalence of severe reactions is high. In the United States, 38.7% of children have a history of severe reactions (Gupta et al., 2011). The most severe food allergic reaction is anaphylactic shock, mediated by massive release of histamine by mast cells, with a contribution of platelet-activating factor (PAF) which is a strong pro-inflammatory lipid mediator and 5-hydroxytryptamine (5-HT,

serotonin) responsible of smooth muscle contraction (Johnston et al., 2014). PAF also induces an increase in vascular permeability and participates in the recruitment and activation of leucocytes (McManus et al., 2000). Anaphylaxis must be treated promptly with an injection of epinephrine. It can be lethal.

The **metabolites derived from arachidonic acid** are prostaglandins and leukotrienes. Leukotrienes are mostly synthesized by the 5-lipoxygenase pathway (Needleman et al., 1986; Hedi et al., 2004). Prostaglandins, resulting from the release of arachidonic acid by phospholipase A2, are produced via prostaglandin endoperoxide synthases. There are four major prostaglandins: prostaglandin (PG) E2 (PGE2), prostacyclin (PGI2), prostaglandin D2 (PGD2) and prostaglandin F2α (PGF2α) to maintain local homeostasis in the body (Ricciotti et al., 2011). Prostaglandins (PGD_2) plays several roles in allergic inflammation, has broncho-constrictive and vasodilating effects, and acts as a chemoattractant for neutrophils (Rasković et al., 1998; Satoh et al., 2006). Leukotriene LTB_4 and cysteinyl leukotrienes (LTC_4, LTD_4, and LTE_4) are involved in a wide variety of inflammatory disorders (Liu et al., 2015). LTB_4 has chemoattractant properties and recruits innate immune cells such as neutrophils, macrophages, and mast cells (Ohnishi et al., 2008). LTC_4, LTD_4, and LTE_4 are produced mostly by eosinophils, mast cells, and macrophages and are primary inflammatory lipid mediators of several inflammatory diseases, including asthma and allergic rhinitis (Liu et al., 2015).

The major **proteases** produced by mast cells are tryptase (tryptic peptidase) and chymase (chymotryptic peptidase) (Caughey, 2007). Among other things, these proteases can damage and activate the bronchial epithelium and contribute to airway wall remodeling (Caughey, 2007; Amin, 2012).

During the immediate reaction, the symptoms vary according to the degranulation site and the concentration of mediators released (Galli et al., 2008). Mast cell degranulation in the gastrointestinal tract increases fluid secretion and peristalsis (the succession of contractions and relaxations of a tubular muscle system) and can provoke vomiting and diarrhea. Mast cell degranulation along blood vessels increases the blood flow and vascular permeability, causing excessive accumulation of fluid in tissues, i.e. edema. Upon respiratory exposure, degranulation in the lung decreases the airway diameters, altering ventilation through airway congestion and blockage (Coico et al., 2015).

In addition, during the sensitization phase, B and T cells migrate into different organs. The allergic reaction thus also depends on the homing site of the B and T cells. For example, T and B cells having localized to the skin will induce atopic dermatitis (eczema), while bronchial asthma will result from B and T cells that have localized to the bronchial tree (Aderbal et al., 2003).

III.IV Cell-mediated hypersensitivity

As shown in Table 1, type IV or T-cell-mediated hypersensitivity can be subdivided into 4 groups. In the first group, macrophages are activated by type-1 helper T cells (T_H1) and cause tissue damage and inflammation initiated by the release of toxic products during phagocytosis (Laskin et al., 2011). In the second group, eosinophils are activated by T_H2 cells and also trigger an inflammatory reaction caused by the release of cytotoxic mediators. In the third group, cytotoxic $CD8^+$ T cells directly cause tissue damage through the release of cytotoxicmolecules (perforin and granzyme) (Coombs, 1963; Andersen et al., 2006; Uzzaman et al., 2012). In the last group, neutrophils are activated by T cells and cause tissue damage induced by the release of toxic products. Symptoms usually occur several hours (6-24 h) after ingestion of the offending food.

Celiac disease is a chronic small bowel inflammatory disorder induced by the malabsorption of wheat, rye and barley (Markiewicz et al., 2012). The soluble protein fractions of wheat the gliadins, and the prolamins in rye and barley which are both rich in glutamine and proline residues, trigger intestinal inflammation in individuals suffering of celiac disease (Arentz-Hansen et al., 2004). The absorptive epithelial cells in the small intestine are damaged by an inflammatory process due to ingestion of certain gluten storage proteins. The abundance of proline residues contributes to the resistance of gliadin and prolamin peptides to gastrointestinal breakdown (Shan et al., 2002). The failure to absorb essential nutrients lead to severe malabsorption symptoms such as diarrhea, weight loss and vitamin deficiencies (Bardella et al., 2005).

Eosinophilic esophagitis (EoE) is a chronic T_H2 inflammatory disease with complex interactions between cells mediating innate and adaptive immunity (Carrard et al., 2015). This chronic disease caused by food allergens is characterized by a severe, isolated eosinophilic infiltration of the esophagus (Liacouras et al., 2005). The release of cytotoxic mediators by eosinophils causes symptoms such as vomiting, regurgitation, heartburn, etc. (Ozdemir et al., 2009; Spergel et al., 2012).

III.V Factors influencing allergic reactions

Worldwide, the number of allergic people is increasing dramatically in different populations. This can be explained by multiple environmental and genetic factors (Lack, 2012). Genetic factors play a crucial role in the development of food allergy (Cochrane et al., 2009). Among the genes proposed to play a role in allergy are those encoding the signal transducer and activator of transcription 6 (STAT6, 12q13),the forkhead box P3 (FOXP3, Xp11.23), expressed in a subset of $CD4^+$ T-cells, and the MHC proteins, which have a crucial role in the immune response (MHC Class II ,6p21)) (Kim,

2009; Tan et al., 2012; Mosaad, 2015; Neeland et al., 2015). A study of 58 twin pairs (monozygotic and dizygotic twins) of which at least one member had peanut allergy has shown a major influence of genetics in peanut allergy, which was found to affect 64.3% of monozygotic but only 6.8% of dizygotic twin pairs (Sicherer et al., 2000).

In developed countries of the world, atopic allergic disease is increasing because of changing environmental factors (exposure to infectious diseases, later exposure to microorganisms) (Janeway et al., 2009; Liu, 2015). The exposure to new molecules such as pesticides (contained in the air, water and/or diet) caused by a more and more polluted environment with a decrease in exposure to infectious agents are considered, in addition to the genetic factors, as the major causative factors of food allergy (Mübeccel et al., 2009). Sex, age, and country of residence also seem to have a major impact on the development of food allergies. As eating habits are different between countries, different allergies tend to appear. For example, mustard seed allergies are prevalent in France, while royal jelly allergies are prevalent in Hong Kong (Nwaru et al., 2014).

Food sensitization can occur at different pre- or postnatal stages. Most studies recommend the avoidance of allergens during pregnancy, but recent studies have shown a protective effect of high consumption of allergens such as peanuts and tree nuts (> 5 times per week) during pregnancy, with a substantial decrease in the rate of allergy developed by the corresponding children (Nowak-Węgrzyn et al., 2017). Another study confirmed that such tolerance might develop, showing a lower probability of cow milk allergy in offspring due to high consumption of milk during pregnancy (800 mL/day) (Tuokkola et al., 2016). On the other hand, however, the early introduction of allergens into the infant diet via breast milk is not recommended and induces food allergy. Clinicians advise the avoidance of peanut until age 3 and of egg, milk, tree nuts, and fish in children less than 1 year old (Goodman et al., 2005; Lack, 2012). The increasing number of allergic children has been found to correlate with the high consumption of peanut butter at an early age in the United States, as compared to the situation in other developed countries where peanut butter is not so popular (Hefle et al., 1996).

As frequently mentioned, alcohol, exercise or drugs can amplify food allergic reactions (Cardona et al., 2012). What is more surprising is that the combination of a specific food and exercise can provoke an allergic reaction, while neither exercise nor food ingestion alone elicits a reaction (Feldweg, 2015). This reaction is called food-dependent exercise-induced anaphylaxis and is characterized mostly by pruritus, urticarial, angioedema, flushing or shortness of breath symptoms (Barg et al., 2011). The mechanism is still not fully understood but exercise might increase the absorption of partially-digested food proteins into the circulation. The proteins migrate into the

tissues, where allergen-specific IgE are fixed on mast cells, triggering the release of mediators such as histamine (Romano et al., 2012).

III.VI Symptoms developed in response to food allergy

Several symptoms that are highly dependent (for their diversity and severity) on the individual and type of allergen can occur within a minute to several hours after the sensitization phase (Skripak et al., 2008). Here is a list of allergic symptoms:

1) **Oral allergy symptoms** (OAS) affect the lips, oral mucosa, and pharynx and usually occur within minutes after contact with the allergen (Amlot et al., 1987; Asero et al., 2007)

2) **Gastrointestinal disorders** occur within a minute to 2 h after allergen ingestion and can provoke nausea, vomiting, abdominal pain, colonic spams, and diarrhea (Asero et al., 2007). Food allergens involved in gastrointestinal disorders are resistant to digestive enzymes (Sampson, 1999).

3) **Skin disorders** are the most common allergic symptoms, characterized by the onset of pruritus, urticaria, and angioedema within a minute to several hours post-ingestion (Asero et al., 2007).

4) **Respiratory disorders** include rhino-conjunctivitis and bronchospasm, especially in patients with allergies to fish, crustaceans, and vegetables (Dannaeus et al., 1977; Asero et al., 2007)

5) **Anaphylaxis** is a severe systemic reaction affecting several organs and due to the release of mediators such as histamine, tryptase, carboxypeptidase A, and proteoglycans from mast cells and basophils. In the majority of anaphylactic reactions, generalized urticaria and angioedema have been observed in 92% of patients (Kemp et al., 2002). A fatal anaphylactic shock is characterized, among other symptoms, by a reduction of blood pressure caused by vasodilation, angioedema induced by an increase in vascular permeability, or breathing problems due to bronchospasms occurring within minutes after exposure to the allergen (Peavy et al., 2008). An injection of epinephrine (0.01 mg/kg) can reverse the anaphylactic shock process, but it has been reported that among 48 lethal cases of anaphylactic shock in the United Kingdom between 1999 and 2006, an injection of epinephrine was provided in 40% of cases (Sampson et al., 2006; Pumphrey et al., 2007). Of the thirty thousand food-induced anaphylactic reactions per year treated by American emergency departments, peanuts and tree nuts are responsible for 80% of the anaphylactic shocks (Burks, 2003). In the United Kingdom, lethal anaphylactic shocks are largely caused (90%) by peanuts and tree nuts (Bock et al., 2001).

IV. Diagnosis and treatment of food allergies

IV.I Food allergy diagnosis

The different diagnostic tests used for food allergy detection are: the skin prick test (SPT), the atopy patch test (APT), oral food challenge (OFC), and antigen-specific IgE testing (sIgE) (**Figure 14**).

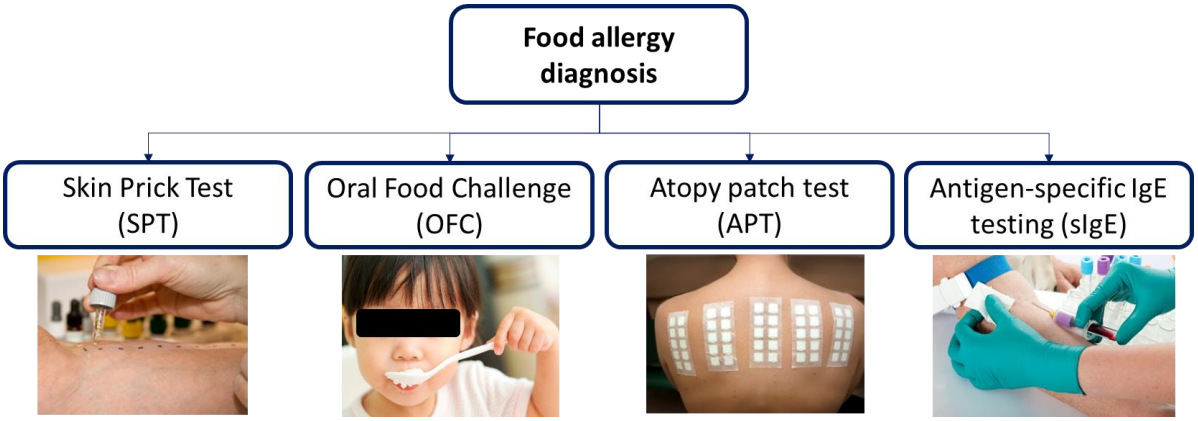


Figure 14: *Techniques used for the diagnosis of non IgE- and IgE-mediated food allergies.*

IV.I.1 The skin prick test (SPT)

The skin prick test is based on recognition of the tested food by specific IgEs presented at the surface of mast cells located in the skin. In the atopic population, an SPT induces mast cell degranulation and mediator release, causing localized vasodilation, angioedemas, and wheals (Mansueto et al., 2006). As negative and positive controls, respectively, exposure to glycerinated saline buffer and to 10 mg/ml histamine are used. These controls are necessary in order to interpret the test. A wheal with a diameter greater than 3 mm is considered a positive result (Salazar et al., 2017). A problem is the high rate of false negatives obtained with commercial SPTs, due to the low abundance or instability of the targeted allergen (Asero et al., 2007; Kumar et al., 2012). In the case of a negative result, the “prick to prick" technique can be used as a confirmatory procedure. This consists in pricking the fresh food (instead of sampling an allergen solution) and then pricking the skin with the same lancet. A negative result obtained with the prick to prick protocol can exclude an IgE-mediated food allergy diagnosis. The SPT is safe, cheap, and easily performed, giving results within 15 min. It is widely used by clinicians for the diagnosis of food allergies. This test, however, has low specificity and leads to a high rate of false positives.

IV.I.2 The atopy patch test (ATP)

The atopy patch test is applied epicutaneously and allows the diagnosis of non-IgE-mediated food reactions (Mansueto et al., 2006). In the allergic population, this test induces eczema skin lesions. Its interpretation is really difficult, however, and finally its use in the diagnosis of food allergies is rather limited.

IV.I.3 Oral food challenge

When an oral food challenge (OFC) is used to diagnose an allergy, a gradually increasing allergen dose must be ingested by the patient under medical observation. The oral challenge can be performed in "a single- or double-blind placebo controlled food challenge" (the latter is abbreviated as DBPCFC) (Asero et al., 2007). The DBPCFC is considered the gold standard for confirming or ruling out the presence of a food allergy. Because the food to be tested is prepared with or without the offending food and because the two preparations are indistinguishable, one avoids drawing erroneous conclusions from the onset of symptoms linked to the stress experienced by an individual during the test (Bock et al., 1988). In preparation for an OFC, the suspected food must be totally excluded from the diet for 7 to 14 days before the food challenge (Mansueto et al., 2006). Furthermore, as a food challenge can induce anaphylaxis, medical staff and equipment must be available. The dangerousness of an OFC excludes patients with a history of severe allergic reactions (Taylor et al., 1992). For these reasons, this test is less used to diagnose food allergies.

In the United States, the FARRP works internationally with clinicians in order to establish DBPCFC protocols for better clinical data comparisons (Mills et al., 2004).

IV.I.4 Specific IgE testing (sIgE)

Levels of antigen-specific IgEs in patient sera are measured with commercial assays. Different methods are used: the radioallergosorbent test (RAST), the enzyme-linked immunosorbent assay (ELISA), and the fluorescence enzyme immunoassay (FEIA) (Salazar et al., 2017). In these assays, antigen bound to a solid support captures the IgE in serum samples. The bound IgE is then recognized by an anti-IgE antibody coupled to either a radioisotope (RAST) or an enzyme catalyzing a reaction whose product can be detected by colorimetry (ELISA) or fluorimetry (FEIA) (Kumar et al., 2012).

Comparisons of the results obtained with different commercial assays have revealed differences due to the lack of harmonization (standards, arbitrary units…). This makes between-study comparisons difficult (Plebani, 2003). The WHO has published a report (WHO/BS/2013.2220) on a

collaborative study aiming to assess the suitability of a lyophilized serum preparation as a 3rd International Standard for serum IgE (Thorpe et al., 2014). The preparation proved suitable, but assay architecture and the quality of allergen extracts and reagents still significantly affect the analytical performance and generate a bias (Plebani, 2003).

Despite costing about three times as much as SPT, sIgE is increasingly used by clinicians for allergy detection, thanks to its sensitivity, specificity, and stability. After the diagnosis of food allergy, patients have to totally exclude the allergenic food from their diet, but clinicians are working on treatments to make the life of allergic patients easier.

IV.II Food allergy treatments

The strict avoidance of food allergens does not always protect food-allergic consumers against inadvertent exposure or cross-contamination during food production (Wang et al., 2013). To avoid severe allergic reactions, clinicians are working on desensitization treatments, their ultimate goal being to repress the immune response and thus promote complete tolerance (Wang et al., 2013). Desensitization leads to the ability to tolerate a food allergen upon continuous exposure to the offending food (Chen et al., 2017).

To determine the efficacy of immunotherapy, many studies enroll two groups of patients: an active group receiving the active substance (e.g. milk proteins) and a control group exposed to a placebo. Two kinds of treatment exist: allergen-specific and allergen-nonspecific immunotherapy (**Figure 15**).

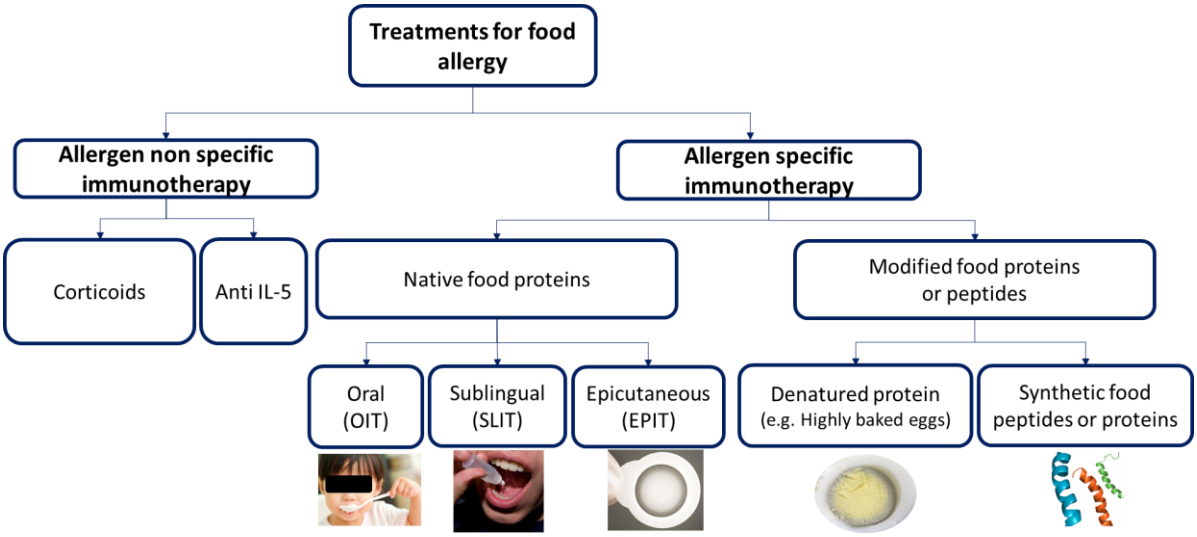


Figure 15: *The most frequent types of treatment used for specific and nonspecific immunotherapies allowing desensitization to allergy-causing foods. Immunotherapy can be administered via the oral (OIT), sublingual (SLIT), or epicutaneous (EPIT) route.*

The mechanism of allergen-specific immunotherapy is based on an immunoregulation through Tregs, cells that have been identified as key regulators of immunological processes (Jutel et al., 2011). Tregs play an important role in the suppression of dendritic cells that support the generation of effector T cells such as: suppression of effector T_H1, T_H2 and T_H17 cells, decrease in allergen specific IgE production and induction of IgG4; inactivation of mast cells, basophils and eosinophils as well as suppression of effector T cell migration to tissues (Akdis et al., 2014; Palomares et al., 2017) (**Figure 16**). Tregs produce IL-10 and TGF, which enhance the production of IgG4 and IgA, respectively by B cells. Immunoglobulins IgG4 are known to block binding of antigens to mast cell and basophil receptors and thus to prevent degranulation and the release of soluble mediators such as histamine, tryptase, carboxypeptidase A, and proteoglycans (Jay et al., 2014). Moreover, during allergen-specific immunotherapy, T_H2 cells, which produce IL-4, IL-5 and IL-13 and induce the activation of mast cells, basophils and eosinophils, are less active (Steveling-Klein, 2016).

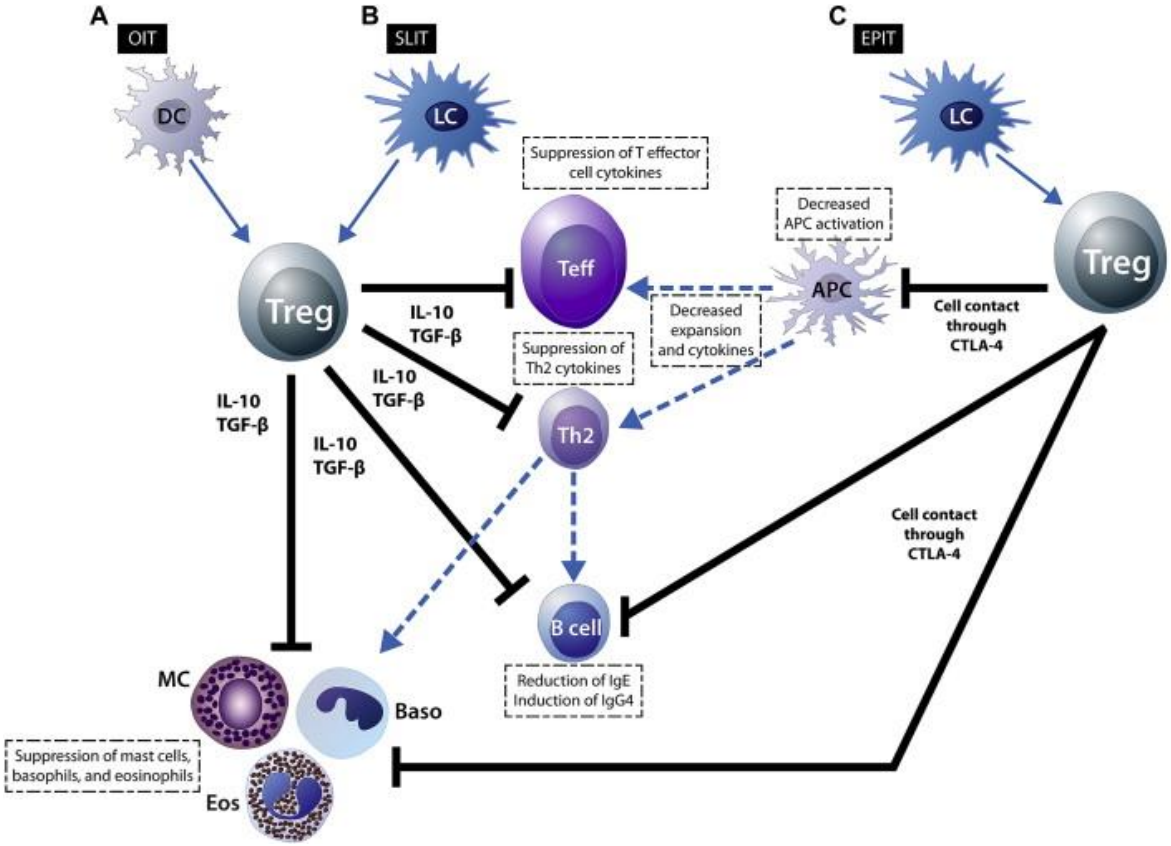


Figure 16: *Putative mechanism of allergen tolerance after specific OIT (dendritic cells), SLIT, or EPIT (Langerhans cells, i.e. dendritic cells of the skin and mucosa). T_H2 cells are suppressed by Treg, through production of interferon IFN-γ and interleukin IL-10, promoting a T_H1 response. Consequently, B cells produce IgG4 instead of IgE, and this limits the activation of mast cells and basophils (from* (Jones et al., 2014)*).*

IV.II.1 Allergen-nonspecific immunotherapy

Nonspecific immunotherapy for food allergy is at an early stage of development. Only two main treatments are briefly described below: traditional Chinese medicine and the anti-interleukin-5 approach.

a) Corticoids

Corticosteroids is frequently used for the treatment of food allergy disorders. However, corticosteroids are not recommended for long-term treatment due to their unavoidable severe adverse and side effects (Patriarca et al., 2009; Zuberbier et al., 2009). T_H2-cell-mediated inflammation is suppressed by corticosteroid, however, this treatment does not influence the history of the disease, even when treatment is started during the childhood (Holgate et al., 2008). Indeed, in a recent study, oral methylprednisolone has been given for 4 weeks to 20 children with eosinophilic esophagitis, and a clinical improvement was observed for the majority of them (19 on 20) (Chehade, 2007).

b) Anti-Interleukin 5

Interleukin IL-5 is a cytokine synthesized and secreted by T_H2 cells and which enhances the recruitment of eosinophils for induction of the late-phase reaction (Bauer et al., 2015). Anti-IL-5 treatment is mostly used to treat asthma, which is a chronic inflammation of the airways characterized by a prominent eosinophilic inflammation (Kay et al., 2004). In a clinical trial, patients with eosinophilic esophagitis (EoE) were treated with anti-interleukin 5 antibodies (mepolizumab). After 4 weeks of two weekly intravenous infusions of 750 mg mepolizumab or a placebo, 54% of treated patients showed a significant decrease in eosinophils, as compared to only 5% of the control group. However, minimal clinical improvement was observed (Straumann et al., 2010). Another comparable study obtained similar results (Dellon et al., 2013).

IV.II.2 Allergen-specific immunotherapy

Allergen-specific immunotherapy is a more developed approach, and promising results have been obtained in the treatment of food allergy, particularly by oral immunotherapy. Whatever the administration route, raw ingredients are used. Modified proteins (baked or synthesized) or peptides, with modifications in the amino acid sequence, are also investigated for allergen-specific immunotherapy, given the dangerousness of ingesting raw ingredients for patients with a history of severe reactions.

a) Oral immunotherapy (OIT)

OIT comprises 3 phases: 1) 6 to 8 doses of allergen are administered in a day; 2) every one or 2 weeks, the patient ingests a dose which is gradually escalated under medical observation until the maintenance dose is reached (6-12 months); 3) a daily home maintenance dose is ingested for years (Wang et al., 2013).

The effectiveness of OIT is supported by several studies (Nurmatov et al., 2012; Yeung et al., 2012; Romantsik et al., 2014). In one study, after OIT, 84% of children allergic to peanuts were able to eat 5 g peanuts, while the placebo-receiving control group tolerated only 280 mg (Nurmatov et al., 2012). In another study on people allergic to milk, consumption of 20 cl milk was possible for 62% of the OIT-desensitized patients, as opposed to only 8% of the control group (Yeung et al., 2012). It is important to note, however, that the protective effect of desensitization is lost or diminished when the treatment is interrupted or discontinued. In a study in which 27 patients received OIT for peanut allergy (dose escalation every two weeks up to 4 g peanut proteins, over a 24-month period), 23 were able to ingest 4 g peanut proteins after desensitization. Then treatment of the 23 desensitized patients was interrupted for 3 months, after which only 30% of them passed the 4 g peanut protein challenge. In addition, after the treatment was stopped for an additional 3 months, only 13% of the patients remained tolerant (Syed et al., 2014).

A higher allergen maintenance dose is used for OIT than for EPIT or SLIT. In addition, OIT appears to be the most effective, with a higher measured concentration of specific IgG4 (Chen et al., 2017). Unfortunately, adverse reactions during OIT, mostly mild, affect 70 to 90% of patients, and some of them require epinephrine administration for severe allergic or anaphylactic-type reactions (Jones et al., 2014; Romantsik et al., 2014). Even though the efficacy of OIT treatment has been proved, for safety reasons it is not suitable yet as a routine treatment against food allergies (Nowak-Węgrzyn et al., 2015).

Nevertheless, Aimmune's drug AR101 is an OIT treatment for peanut allergy in the final stages of clinical testing to support regulatory approval. The amount of peanut proteins in AR101 treatment is from 3 mg to 300 mg, which is much lower than the 4000 mg peanut proteins previously reached after 24 months. This makes the new treatment safer (Thorpe et al., 2014). In phase II, AR101 was tested on highly reactive patients (<100 mg peanut proteins). The starting dose of AR101 was 3 mg per day, with updosing every two weeks in hospital, up to 300 mg per day after 22 weeks. Of the 29 patients receiving the active substances, 6 were excluded from the trial because of gastrointestinal side effects. Of the remaining 23 patients, 62% were able to pass the challenge with 1.043 mg peanut proteins (4 peanuts), while the entire control group receiving a placebo failed (Kingwell,

2016). Medical supervision every two weeks should be provided for the dose escalation, but AR101 could change the quality of life (stress, social life) of highly allergic patients.

b) Sublingual immunotherapy (SLIT)

SLIT is similar to OIT, but the gradually increasing doses of allergens are placed under the tongue (Le et al., 2014). The maintenance dose is 1000 times lower for SLIT than for OIT, because of the small amount of allergen that can be placed under the tongue (Moran et al., 2013). After 12 months of SLIT, patients desensitized to peanut allergens were able to consume 1710 mg peanut proteins, while the control group could only ingest 85 mg without developing symptoms (Kim et al., 2011 a). While OIT is more effective than SLIT for food allergen desensitization, SLIT is safer, thanks to the smaller amount of allergens ingested by patients (Jones et al., 2014). The long-term efficacy of both treatments is still unknown, but desensitization can be lost within a week after interruption of the treatment (Wang et al., 2013).

c) Epicutaneous immunotherapy (EPIT)

An EPIT desensitization study for peanuts, performed for 12 months, involved daily application of Viaskin patches (250 µg peanut proteins) on the skin. For the phase II clinical trial, highly reactive child and adult patients, supporting doses lower than 300 mg peanut protein in an oral food challenge test, were selected. After 12 months of EPIT peanut desensitization, 32.1% of the patients were able to pass the 1 g peanut protein challenge, while only 12.5% of the placebo-receiving control group passed this test (Mondoulet et al., 2015; Kingwell, 2016). In the phase III trial, however, the statistical significance of the difference between treated patients and controls was not sufficient to consider the trial successful. Hence, it has not yet been possible to commercialize Viaskin.

EPIT seems to be the least effective allergen-specific treatment as compared to OIT and SLIT, but in terms of safety, only local skin reactions such as eczema have been reported (Chen et al., 2017). The quantity of allergens in a skin patch is limited and cannot readily be increased (Senti et al., 2014).

Specific immunotherapy has led to better desensitization of allergic patients than nonspecific immunotherapy. Yet the high number of adverse reactions observed during treatment, such as vomiting, abdominal pain, and anaphylactic reactions, have prompted researchers to look for safer approaches, such as treatments with modified proteins (baked or synthesized) or peptides. It is noteworthy that despite the promising results of AR101 treatment, 20.7% of patients (6 out of 29)

were excluded from the trial. Hence, alternative treatments must be found for patients with severe allergy.

d) Highly baked allergens

Proteins possess conformational and sequential epitopes (**Figure 1**). Conformational epitopes are lost upon baking, because of protein denaturation (Sampson, 2004).

Consequently, some proteins (such as caseins in milk and ovalbumin or ovomucoid in eggs) become less allergenic upon baking. Clinical trials performed with highly baked milk and eggs have revealed that between 70 and 80% of children can safely eat baked egg and milk products, as the conformational epitopes are no longer recognized by specific IgE antibodies (Chen et al., 2017). Furthermore, consumption of heated eggs and milk could be a way to induce a tolerance. In one study, highly baked milk (1.3 g milk protein in muffin cooked at 177 °C for 30 min) was tolerated by 68 milk-allergic children out of 100, and after three months of daily consumption of baked milk the SPT wheal diameter was significantly smaller and the level of casein-specific IgG4s significantly higher, proving that consumption of highly baked milk can engender tolerance (Nowak-Wegrzyn et al., 2008). These results were confirmed in a study on egg-allergic patients, where an increase in ovalbumin- and ovomucoid-specific IgG4s was observed after consumption of highly baked egg (Lemon-Mulé et al., 2008). Sequential epitopes, however, are resistant to protein denaturation and can result in a more severe reaction after food baking (Husain et al., 2013). What's more, chemical modifications induced at high temperature by Maillard reactions could induce formation of new conformational epitopes in some proteins, as demonstrated for Ara h2 (Beyer et al., 2001). Accordingly, IgE binding to the peanut proteins Ara h1, Ara h2, and Ara h3 in allergic patient sera was significantly higher with roasted peanuts (170 °C-20 min) than with raw peanuts (Maleki et al., 2000 b; Beyer et al., 2001).

Hence, this treatment cannot be applied to all allergens, but it could be suitable for milk and egg allergies.

e) Recombinant peptide and protein immunotherapies

Recombinant peptide and protein immunotherapies have been made possible by recent advances in molecular biology, recombinant DNA technology, purification, and sequencing/cloning technology, allowing modification of peptides and proteins for allergen-based immunotherapy (Cook et al., 2018).

Scientists have synthesized soluble peptide fragments of T-cell-targeted epitopes (8 -16 amino acids) showing optimal MHC class II binding but which are too short to cross-link IgEs and activate

mast cells and basophils (Wang et al., 2011). In a study devoted to peanut allergen Ara h2 in a mouse model, five dominant T-cell epitopes were identified and three short peptide fragments were synthesized. The study demonstrated that the synthetic peptides could bind to the T-cell $CD4^+$ receptor and that the fragments were too short to cross-link IgEs on mast cells and basophils (Chen et al., 2017; Reisacher et al., 2017). For this therapeutic approach, T-cell epitopes must be identified for each allergen.

For recombinant protein immunotherapy, IgE binding sites must be identified on each allergen in order to synthesize, from allergen-encoding DNA, purified proteins with a disrupted IgE binding site (Valenta et al., 2016). This disruption favors a robust IgG antibody response by preventing IgE binding to the altered allergen. It avoids cross-linking of specific IgE on mast cells and basophils and hence the release of soluble mediators such as histamine, proteases, etc. (Reisacher et al., 2017).

Peptide and recombinant protein immunotherapies are at an early stage of development and are performed only on sera from allergic patients. The tolerance-inducing mechanism is not fully understood and must be clarified.

Section II: Food allergen labeling and regulations

V From food allergen thresholds towards safe food products

V.I Clinicians and (or versus) authorities: determining thresholds

Despite an increasing number of clinical trials, European public health authorities have established thresholds only for sulfites (10 mg/kg) and gluten (20 mg/kg for "gluten free" and 100 mg/kg for "very low gluten"), in Regulations (Regulation No. 1169/2011, 2011; Regulation (EU) No 828/2014, 2014). Thresholds for the main allergens were first set in Switzerland and Japan, a long time ago (Taylor et al., 2014 a). In April 2002, Japan was the first country to mandate allergen labeling under a national law. The introduced labeling was based on the actual illness and degree of seriousness of allergic reactions and the law stipulated for seven allergens (egg, milk, wheat, buckwheat, peanut, shrimp, and crab) that they be declared on the labels of food products containing more than 10 mg allergen proteins per kg (Akiyama et al., 2011).

In Switzerland, the federal department requires labeling of allergens present at concentrations above 1000 mg ingredient per kg of food products (Crevel et al., 2008). Yet studies based on clinical data have determined 11 thresholds for the major allergenic foods, ranging from 0.03 mg for egg proteins to 10 mg for shrimp proteins (Taylor et al., 2014 a). The legal threshold set by Swiss law is really high, and does not prevent severe allergic reactions from occurring. Thresholds are expressed in absolute quantity (mg of proteins, mg of ingredients) or even as doses (mg of proteins or ingredients per kg of food products), which make difficult the comparison of thresholds between countries. Indeed, conversion factors exist but the percentage of proteins can change between materials used (e.g. milk, skimmed milk powder, casein proteins, milk powder). Moreover, the percentage of proteins in 1000 mg of milk will be totally different than 1000 mg of milk powder both used during food production which can be complicated/confusing for industrials.

V.I.1 Large-scale clinical studies

A threshold dose is commonly defined as the lowest amount of allergen that can induce mild allergic symptoms in most sensitive individuals after a food challenge (Taylor et al., 2002). The Swiss and Japanese thresholds were established without taking into account the No Observable Adverse Effect Levels (NOAELs) and Low Observable Adverse Effect Levels (LOALs) obtained from clinical food challenges on food allergic subjects (Taylor et al., 2014 a). Setting inappropriate thresholds

without a large-scale study based on scientific and clinical data can endanger the food-allergic population.

In 2011, to tackle the lack of legal thresholds in most countries, a panel of experts was formed in the framework of the VITAL program (Voluntary Incidental Trace Allergen Labeling program) of the Allergen Bureau of Australia & New Zealand. This program establishes reference doses and helps industrialists evaluate potential risks of cross-contamination during food production (Taylor et al., 2014 a, 2015).

VITAL has established eliciting doses (EDs) on the basis of NOAELs and LOAELs obtained from clinical studies all over the world. The eliciting dose ED_p is the quantity of allergen, expressed in mg total proteins, that is likely to induce an allergic reaction in a percentage p of the allergic population (Crevel et al., 2014). For milk, egg, and hazelnut allergens, an ED_{01} has been determined, i.e. an amount of allergen likely to cause a reaction in only 1% of allergic individuals. The robustness of an ED_{01} can be determined only when data are available for more than 200 clinical patients challenged with the antigen (Taylor et al., 2014 a). An ED_{05} has been determined for wheat, mustard, lupine, cashew, sesame, shrimp, and fish. Food challenge studies, however, exclude individuals with a history of life-threatening reactions. This causes a bias hindering determination of a true ED (Crevel et al., 2008).

The “gold standard” for determining LOAELs is the double-blind placebo-controlled food challenge (DBPCFC), in which patients receive on different days the same food preparation with and without the offending allergen. Basically, patients receive at intervals an escalating dose (typically from 3 mg to 3 g food proteins) of the allergenic food (Muraro et al., 2014). Although VITAL has set thresholds for the major allergens, they are not yet accepted as legal thresholds. The severity of an allergic reaction varies according to the individual, the allergens and their industrial processes (e.g. thermal process, freezing, pasteurization) , but physical exercise, alcohol, and acute infections can also influence the severity of a food allergy, making it hard to establish legal thresholds (Niggemann et al., 2014).

VI.I.2 Different thresholds in different countries

The Netherlands Food and Consumer Product Safety Authority (NVWA, Nederlandse Voedsel- en Warenautoriteit) has also set thresholds based on zero risk for allergic individuals. The NVWA considers that the ED_{01} and ED_{05} determined by VITAL are too high, as 2 to 3% of the allergic population can still have mild reactions at these threshold doses. While VITAL has set thresholds ranging from 0.03 mg for egg proteins to 10 mg for shrimp proteins, the NVWA has set thresholds for these allergens at 0.0043 and 3.7 mg, respectively (NVWA, 2016). Yet to ensure zero risk, an

analytical detection method should be able to measure doses corresponding to these thresholds. To date, none of them is sensitive enough to do so, especially for detection in processed food products (see Section 3). In addition, Madsen *et al.* (2012) have declared zero risk to be unrealistic (Madsen et al., 2012), as threshold levels vary from one individual to the other and also, for a given individual, in response to different exposures. Consequently, it is difficult for the food industry to comply with zero risk criteria, as several sources of contamination share the same production line for different products or because of volatile dusts (e.g. flour and milk powder), etc. (Kerbach et al., 2009)

A panel of experts has highlighted that a risk-based approach should be performed by implementing a tolerance threshold for each allergen and conducting a large-scale trial at the tolerance threshold concentration to obtain additional data (Madsen et al., 2012). The industry could use these thresholds as risk management decision tools for allergen labeling and to justify food product recalls (Madsen et al., 2012).

The scientific comity of the Federal Agency for the Safety of the Food Chain (SciCom of FASFC) has chosen to respect the ED_{05} of VITAL, while Germany has chosen to use VITAL thresholds (**Table 2**) (SciCom, 2017; Waiblinger et al., 2017).

Food	Reference dose (VITAL)	Reference dose (EAACI)	Reference dose (NVWA)	Reference dose (SciCom, FASFC)	Reference dose Germany
Peanut	0.2	0.2	0.015	1.1	0.2
Cow milk	0.1	0.1	0.016	1.2	0.1
Egg	0.03	0.03	0.0043	0.3	0.03
Hazelnut	0.1	0.1	0.011	0.5	0.1
Soy	1.0	1.0	0.078	2.9	1.0
Wheat	1.0	1.0	0.14	1.3	1.0
Cashew	2.0	2.0	1.4	0.6	2.0
Mustard	0.05	0.05	0.022	0.1	0.05
Lupine	4.0	4.0	0.83	4.5	4.0
Sesame	0.2	0.2	0.10	0.4	0.2
Shrimp	10.0	10.0	3.7	12.1	/

Table 2 : *Reference doses for different food allergens according to VITAL (the Voluntary Incidental Trace Allergen Labeling program)* (Allen et al., 2014 a; Taylor et al., 2014 b)*, the EAACI (European Academy of Allergology and Clinical Immunology)* (Muraro et al., 2014)*, NVWA (Netherlands food and consumer product safety authority)* (NVWA, 2016)*, FASFC (Federal Agency for the Safety of the Food Chain)* (SciCom, 2017)*, and Germany* (Waiblinger et al., 2017).

It also worth stressing is the fact that thresholds in Europe differ greatly from country to country. For example, legal thresholds for peanut allergens range from 0.015 mg peanut proteins in the Netherlands to 1 g peanut per kg (corresponding to 250 mg peanut proteins) in Switzerland. The threshold differences between European countries will be even more complicated for industrialists and will create many difficulties for products exported to different countries.

To ensure the safety of allergic individuals and to respect these thresholds, the food industry needs to limit (as much as possible) contaminations during food production. In addition, it must perform risk assessments to evaluate the risk of adverse reaction development in the allergic population.

V.II The food industry: managing cross-contamination

V.II.1 Cross-contamination in food establishments

Generally, the most severe allergic reactions occur in restaurants or other food establishments (Taylor et al., 2006). A study of 106 allergic reactions having taken place in restaurants or similar establishments (ice cream shops, bakeries) showed that 45% of the reactions happened even when the allergic people gave clear instructions, and in 27% of cases the reactions were very severe (Furlong et al., 2001). The prevalence of allergic reactions in food establishments leads to major social exclusion and stress for allergic customers, especially since 76% of fatal anaphylaxis events due to food happen away from home (Gowland, 2001).

During food production, cross contamination by food allergens can occur at every step: food suppliers, food storage, shared equipment…(Taylor et al., 2010; Allen et al., 2014 b). The absence of any indication on the packaging is reported to be responsible for 85% of product recalls due to food allergens in the European Union (Rapid Alert System for Food and Feed RASFF), 60% in the United States (Food and Drug Administration FDA), and 86% in Canada (Canadian Food Inspection Agency CFIA) (Bucchini et al., 2016). Although the management of cross-contamination by industrialists is increasingly strict, zero risk for allergic consumers can never be reached.

One way to improve the safety of food products is for industrialists to evaluate the risk of contamination. Assessing risks means evaluating the probability that an adverse reaction will occur after exposure to a food product (Spanjersberg et al., 2007; Kruizinga et al., 2008; Madsen et al., 2012). To promote food safety, the European Union recommends the hazard analysis critical control point (HACCP) approach described in Regulation 2004/852/EC (Bryan, 1990; Regulation (EC) No 852/2004, 2004; Newslow et al., 2005; Hurst, 2013). This approach is based on traceability, on separating allergenic from other ingredients, and on cleaning supply chains under both visual and analytical control (Ward et al., 2010). The lack of systematic risk assessment in food supply chains

has led, however, to abusive use of precautionary allergen labeling (PAL) and finally to a lack of suitable information for allergic consumers (Crevel et al., 2008).

VI.II.2 Food allergen labeling

Food labeling must provide complete information about food content (Cheftel, 2005). Indeed, allergic consumers have to completely exclude the allergenic food from their diet, because currently there exist only rescue therapies to counter anaphylaxis reactions (DunnGalvin et al., 2015). Two kinds of allergen labeling can be found: mandatory (required by authorities) and precautionary (established by industrialists).

a) Mandatory labeling

Worldwide, significant differences in food allergen labeling regulations are noticeable. The Codex Alimentarius Commission (CAC) is an organization formed by the Food and Agricultural Organization (FAO) and the World Health Organization. In 1999, the CAC adopted a list of priority allergens. The priority allergens determined by the CAC are responsible for the vast majority of allergic reactions. Some countries or regions, however, have decided to modify this list (Taylor et al., 2015) (**Table 3**).

The Food Allergen Labeling and Consumer Protection Action (FALCPA) in the United States requires labeling of only 8 food allergens: milk, egg, peanut, tree nuts, fish, crustacean shellfish, wheat, and soy, instead of the 14 required by the European Union (Kim et al., 2011 b). Between-country differences in targeted allergens depend on the prevalence of allergic reactions. For example, because mustard is widely consumed in Europe, it is mandatory to declare it on food labels in the European Union.

In Europe in the 1990s, only ingredients incorporated into a recipe in proportions exceeding 25% were indicated on labels (Bruijnzeel-Koomen et al., 1995; Eigenmann, 2001). Authorities having become aware of the difficulties caused by this labeling, Directive 2000/13/EC made it mandatory to label ingredients representing more than 5% of the recipe/total content. Nowadays, it is mandatory to mention on food labels the presence of 14 allergens, when they are introduced as ingredients into the food product (Bruijnzeel-Koomen et al., 1995; Directive 2000/13/EC, 2000; Mills et al., 2004).

Allergen regulations, however, do not take into account the “hidden allergens” resulting from cross-contamination during food production. These can also cause severe reactions (Mills et al., 2004).

	Codex Alimentarius Commission	U.S.	EU	Canada	Australia/ New Zealand	Japan	Korea
Milk	X	X	X	X	X	X	X
Eggs	X	X	X	X	X	X	X
Peanut	X	X	X	X	X	X	X
Gluten	X		X	X	X		
Wheat	X	X	X	X	X	X[a]	X[b]
Crustacea	X	X	X	X	X	X[a]	X[b]
Fish	X	X	X	X	X		X[b]
Soybean	X	X	X	X	X		X
Tree nuts	X	X	X	X	X		
Sesame seed			X	X	X		
Molluscs			X	X			
Mustard			X	X			
Celery			X				
Lupine			X				
Buckwheat						X	X
Other						X[a]	X[b]

[a] Japan: Shrimp and crab are the only Crustacea on the list. Grains include wheat and buckwheat but not other cereal sources of gluten. Other includes foods that are on a recommended but not required labeling list including salmon, salmon roe, mackerel, abalone, squid, beef, pork, chicken, soybean, orange, kiwi, banana, peach, apple, yam, gelatin, matsutake mushroom and walnut.
[b] Korea: Shrimp and crab are the only Crustacea on the list. Grains include wheat and buckwheat but not other cereal sources of gluten. Other includes peach, pork and tomato.

Table 3: *Priority list of food allergens defined by the Codex Alimentarius Commission (CAC) in 1999 and its implementation in the United States, the European Union, Canada, Australia/ New Zealand, Japan, and Korea* (Taylor et al., 2015).

b) Precautionary allergen labeling

Precautionary Allergen Labeling (PAL) is used by the food industry to provide better information for the allergic population and to avoid expensive food recalls. Several risk sentences can be read on food packaging: “may contain…”, “produced in a factory which handles…”, “made in a production area that also used…”(Kim et al., 2011 b). The use of PAL is prohibited in Japan and Argentina, regulated in South Africa, and widely used in the rest of the world (Allen et al., 2014 b). While PAL can be considered helpful to allergic people, its use is not systematically associated with a risk assessment, and it ultimately misleads consumers.

In one study, investigators analyzed 569 cookies and chocolates from 10 European Union countries for the presence of hazelnuts and peanuts, comparing products with and without PAL. In chocolates, hazelnuts were detected in 79% of PAL products and 53% of non-PAL products. Peanuts were detected in 25% of PAL products and 11% of non-PAL products. In cookies, the percentages of hazelnut- containing and peanut-containing products were respectively 36% and 43% for PAL products, and 25% and 25% for products without PAL. This study clearly highlights the poor

relationship between PAL and the presence of the targeted allergen (Pele et al., 2007). PAL is used by food suppliers as a “safety net”, because extensive cleaning and respecting manufacturing protocols cannot totally eliminate contaminations (DunnGalvin et al., 2015).

In a large study in Canada (1454 allergic individuals), accidental exposure to a food allergen was reported by 47.8% of participants. It was principally caused by inappropriate labeling (47%), failure to read existing food labeling (28.6%), or ignoring PAL (8.3%) (Sheth et al., 2010). The poor relationship between PAL and the presence of allergens leads customers to consume PAL products and thus risk developing allergic reactions (DunnGalvin et al., 2015).

To improve the life conditions of allergic people, food laboratories are working on improving allergen detection and quantification to allow better labeling.

Section III: Food allergen analysis

VI Analysis of food allergens by laboratories

VI.I Analysis of allergens in foodstuffs

The last section of this introduction is dedicated to the methods used to analyze allergens by mass spectrometry. Briefly, the methods most used at the present time to detect allergens are the ELISA and polymerase chain reaction (PCR) methods. Yet as discussed here and in a press article (LabInfo n°16 AFSCA - Annex 1), allergen detection in processed food is extremely difficult and leads to false negatives.

VI.I.1 Detection of proteins by ELISA

Enzyme linked immunosorbent assay (ELISA) is the most widely used technique to analyze food allergen proteins in foods because of its sensitivity, specificity, rapidity and the low cost of analysis (Asensio et al., 2008). The serum of immunized animal containing allergen specific immunoglobulin G (IgG) is used (Montserrat et al., 2015).

Sandwich immunoassay is the assay format often used for the analysis of food proteins (**Figure 17**). This method uses two antibodies, a capture and a detection antibody, which can bind to different epitope sites on the allergen. The capture antibody is highly specific for the allergen and attached/coated to a solid surface. The protein extract of the food product to test is then added and followed by the addition of the conjugated detection antibody to an enzyme. The enzymatic activity is measured (e.g. goat, anti-rabbit IgG). A colorimetric reaction is catalyzed by the enzyme in the presence of appropriate substrates and the absorption of the product that accumulates overtime is proportional to the amount of allergen fixed by the capture antibody, allowing the quantification of allergens.

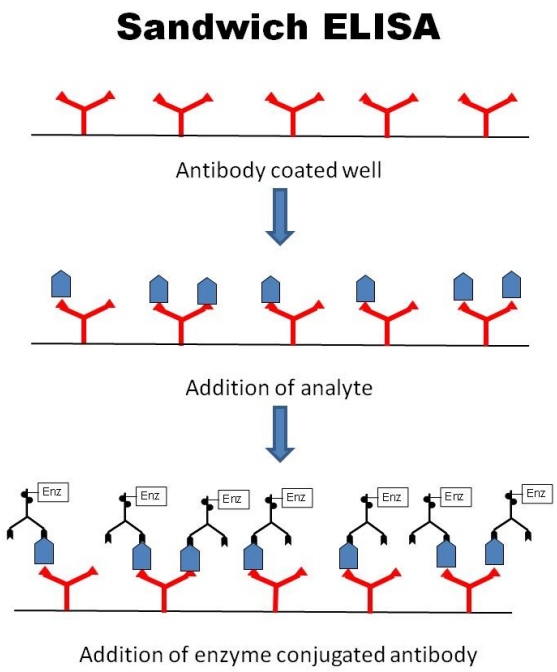


***Figure 17**: Diagram of a sandwich ELISA. The addition of the enzyme's substrate leads to a colorimetric reaction. The absorbance of the product formed is directly proportional to the allergen concentration.*

The main advantages of ELISA is the price and the rapidity to analyze proteins with sensitivity and specificity in food products (Montserrat et al., 2015). However, the epitope of the allergen that is recognized by the antibody can be linear or conformational and the later can be lost during the food process. ELISAs are thus not able to accurately determine the amount of proteins present in thermally processed foods due to changes in the extractability, solubility and immunoreactivity such as a loss of conformational epitope or aggregations of the target proteins (Fu et al., 2013). In addition, a lack of specificity of antibody can lead to cross-reactions with closely related proteins and thus, to false positives (Kirsch et al., 2009). Moreover, the quantification of allergens, especially in processed foods obtained by different ELISA tests, gives significantly varying results due to the use of different antigens, antibodies and assay formats (direct, indirect and sandwich). Moreover, the variability of IgE/IgG ratio between sera will enhance variability due to a competition between IgE and IgG for binding to the same allergen (Montserrat et al., 2015).

VI.I.2 Detection of DNA by PCR

The polymerase chain reaction (PCR) and real-time PCR are DNA-based methods in which specific DNA fragments are amplified by PCR (**Figure 18**). Allergenic ingredients in processed foods can be more easily detected by PCR, because DNA is less affected by thermal treatments than proteins

(Linacero et al., 2016). The specificity of PCR is achieved by the use of primers that allows amplification of DNA fragments originating from the allergen.

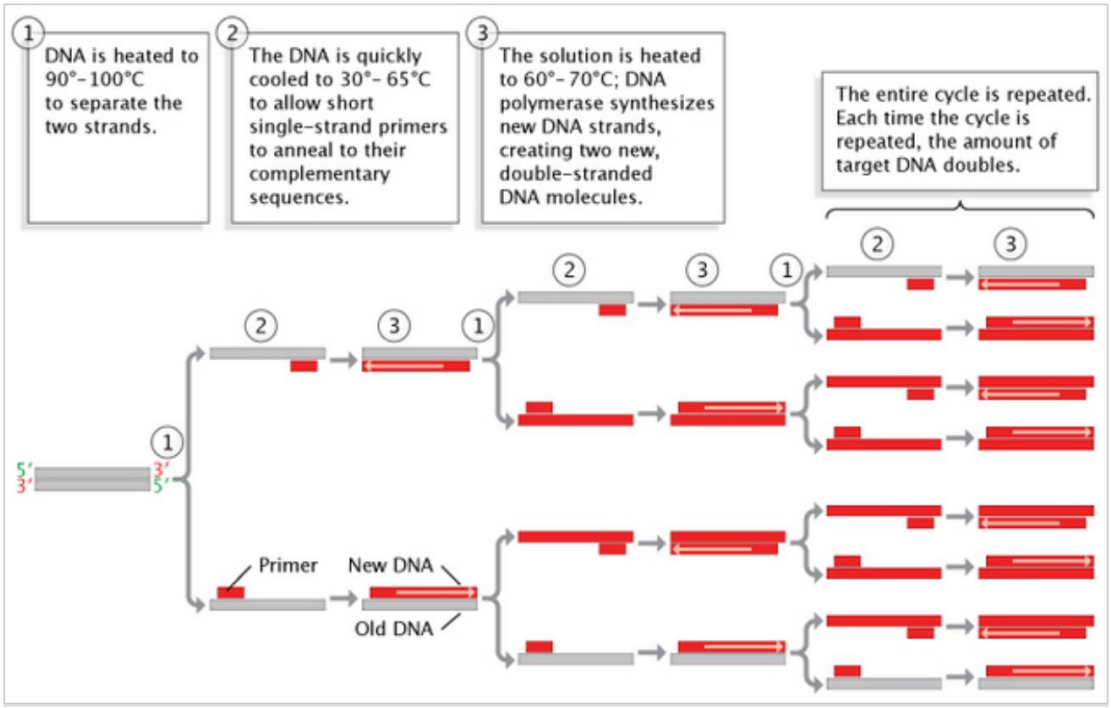


***Figure 18**: The different phases of a polymerase chain reaction (PCR): (1) denaturation, (2) primer annealing and (3) primer extension and amplification of DNA (from* (Pray, 2008)*).*

The main limitation of DNA-based methods is the fact that allergenic proteins are not targeted, but only the DNA which can only determine the taxonomy (e.g. no difference between a chicken and an egg origin) leading to many false positives and indeterminations (Van Hengel, 2007; Kirsch et al., 2009).

VI.I.3 Detection of peptides by mass spectrometry

Mass spectrometry is more and more used for the detection of peptides from allergenic proteins. The main advantages of mass spectrometry is it high sensitivity, specificity and the possibility to analyze several allergens in a single analytical run (Boo et al., 2018). Moreover, mass spectrometry targets peptides. Consequently, processed and unprocessed food allergens can be analyzed (Heick et al., 2011; Planque et al., 2017 a). A loss of sensitivity is observed for the detection of peptides in processed food products by mass spectrometry but the effects of the process observed by mass spectrometry are not so severe than for most ELISA kits (Pilolli et al., 2018). The principle of an analysis by UHPLC-MS/MS in MRM mode is described in the **Figure 19**.

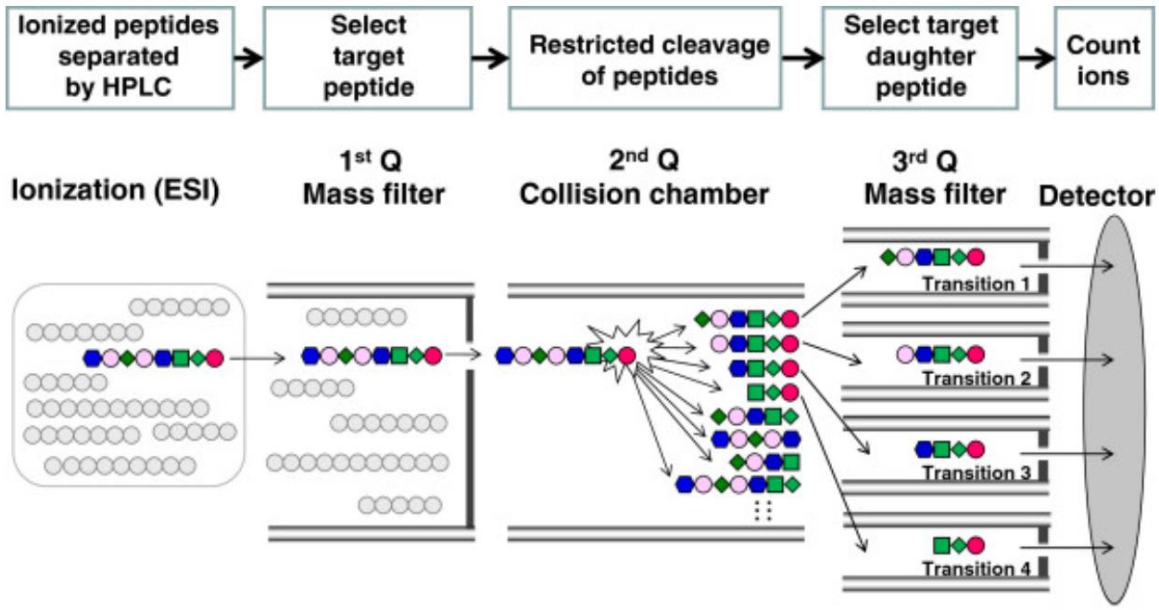


***Figure 19**: Principle of peptide selection by triple quadrupole mass spectrometry in multiple reactions monitoring (MRM) mode. The target peptide is selected in the first quadrupole, fragmented, and fragments are selected in the third quadrupole. The mass to charge (m/z) ratio of the target peptide selected in the first quadrupole and of the fragment selected in the third quadrupole is called a transition (e.g. FFVAPFPEVFGK 692.9>920.5) (from* (Uchida et al., 2013)*).*

The main disadvantage of mass spectrometry is the cost per analysis. Consequently MS is mostly used as a confirmatory tool for ELISA or PCR data generated in routine laboratories.

Some of the modifications that can affect allergens and influence/impair their detection by mass spectrometry are presented below, before a book chapter on "Food Allergen Analysis: Detection, Quantification and Validation by Mass Spectrometry" published in the InTechOpen Allergen Book (http://dx.doi.org/10.5772/intechopen.69361).

VI.II Modifications of food allergen proteins

During food processing, proteins can be unfolded, aggregated and chemically modified (Mills et al., 2009). Proteins could interact and or react with each other and with metabolites, phospholipids, carbohydrates and nucleic acids leading to post translational modifications (Nørregaard Jensen, 2004). For example, the phosphorylation of a serine residue leads to a mass increment of 80 Da, thereby increasing the nominal molecular mass of this residue from 87 Da to 167 Da (Mills et al., 2009). Consequently, the peptide cannot be detected by UHPLC-MS/MS anymore. The most important post translational modifications are represented in the **Figure 20.**

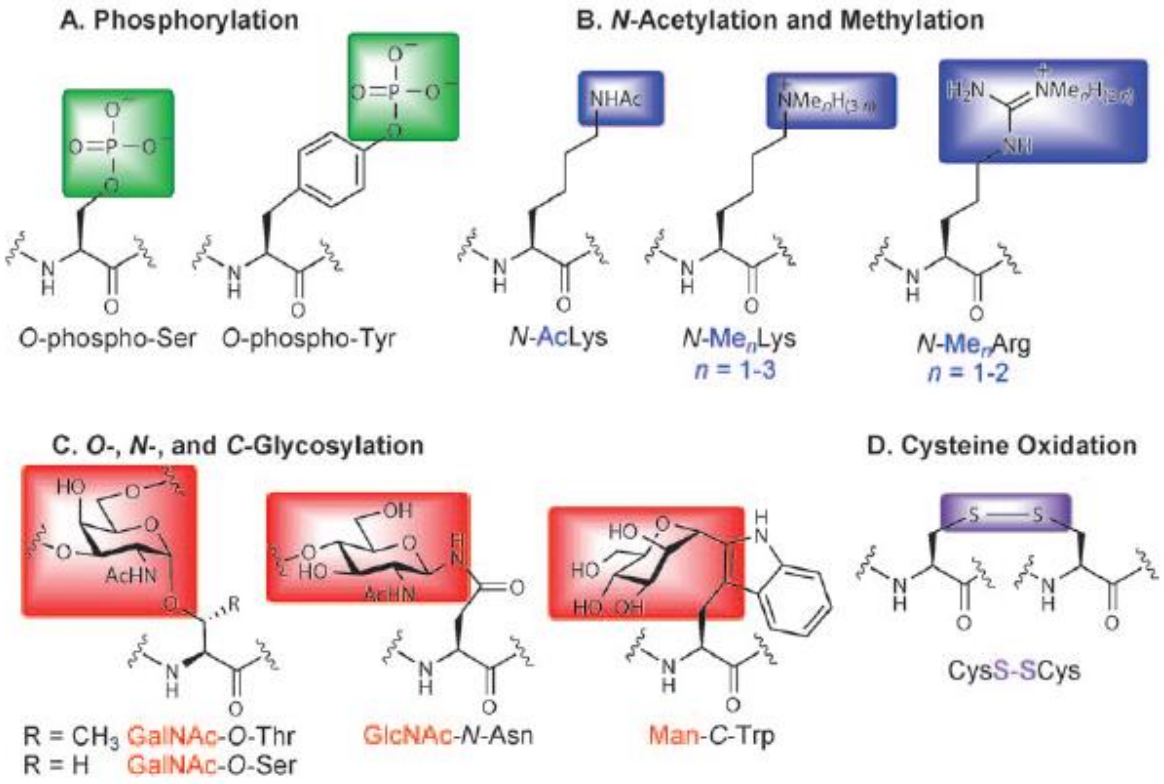


Figure 20: *Most common post translational modifications of proteins: phosphorylation, N-acetylation, N-glycosylation and oxidation (modified from* (Chalker et al., 2009)*).*

Food processing often leads to protein crosslinking, referring to the formation of disulfide bonds formed by the oxidative coupling of two cysteine residues. The formation of disulfide bonds is the most common covalent crosslink, between polypeptide chains within a protein or between proteins (Gerrard, 2002).

The native structure of a globular protein is highly organized and first determined by a primary structure consisting of a combination of 22 amino acids with hydrophilic or hydrophobic properties. For example, the amino acids isoleucine (Ile, I), leucine (Leu, L), and valine (Val, V) have long aliphatic chains, and phenylalanine (Phe, F) and tryptophan (Trp, W) have aromatic chains. Such chains confer hydrophobic properties to the protein. The secondary structure consists of α-helices and β-sheets arising through hydrogen bonding between amino and keto groups in the polypeptide chain (**Figure 21**) (Davis et al., 1998). The three-dimensional shape of the protein (conformation) is determined by noncovalent interactions such as hydrogen bonds (between polar groups), ionic interactions (between charged groups), hydrophobic interactions (among nonpolar groups in aqueous solution), and van der Waals interactions or London forces (opposite electric dipole attractions) (Buxbaum, 2015). The quaternary structure is formed by association of several polypeptide chains through ionic and hydrophobic interactions between amino acid side chains.

Hydrophobic groups lie on the inside of the structure, while hydrophilic groups are on the outside, where they interact with water on the surface of the protein (Davis et al., 1998).

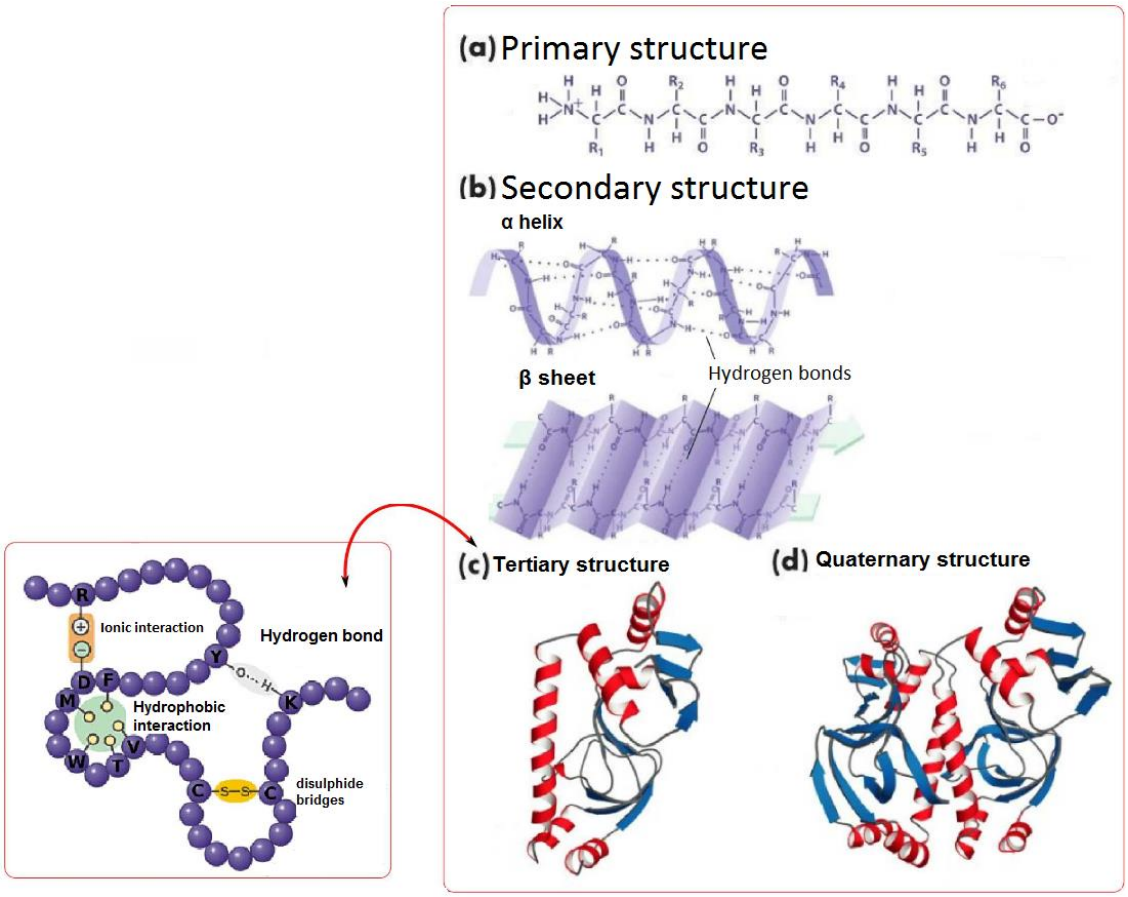


Figure 21: *Structure of a globular protein with hydrophobic and ionic interactions, hydrogen and disulfide bridge (modified from* (Férey et al., 2009)*).*

When an allergen is denatured by a thermal process, the quaternary and tertiary structure and to some extent the secondary structure of the native proteins are altered (Mills et al., 2009). At a temperature around 80 °C, the secondary and tertiary structures of the proteins are almost lost, and the proteins adopt a fully unfolded random conformation.

One frequent effect of heating concerns disulfide bonds, i.e. covalent bonds which constitute crosslinks within or between protein chains (Singh, 1991). A disulfide bond is formed through oxidative coupling of two cysteine residues: the hydrogen atoms of the thiol groups (-SH) of the two cysteines are removed by oxidation and a bond is formed between the two sulfur atoms (Gerrard, 2002). At 80 °C, disulfide bonds are cleaved and new inter- and intra- protein disulfide bonds can be formed (Gerrard, 2002; Wal, 2003). During thermal treatment of milk, for example, a disulfide bond is formed between β-lactoglobulin and κ-casein (Singh, 1991). Moreover, hydrophobic groups

normally buried become exposed, thus altering the hydrophobicity of the protein surface (Mills et al., 2009).

Secondly, at about 90-100 °C, aggregates are formed. Protein aggregation can involve several types of physical interaction, including van der Waals, hydrophobic, and electrostatic interactions, hydrogen bonds, and disulfide bonds (Wal, 2003; Mills et al., 2009). These different interactions are influenced by factors such as the pH, the protein concentration, and whether and for how long thermal processing is carried out. For example, a pH near the isoelectric point (pI) of a protein promotes its aggregation, due to the close-to-zero net charge (Gerrard, 2002).

At temperatures above 100 °C, chemical modifications can occur. The most important and prevalent chemical reactions that take place during thermal processing are the so-called Maillard reactions (Davis et al., 1998; Mills et al., 2009), i.e. reactions between free amino groups on proteins and groups on reducing sugars (aldehyde groups (−CHO) on sugars such as glucose and maltose or ketone groups (-CO-) on sugars such as fructose).

More precisely, Maillard reactions are characterized by formation of a Schiff base through the reaction of a reducing sugar with a free amino group, particularly an ε-amino group of lysine or the α-amino group of a terminal amino acid (Feeney, 1977; Halford et al., 2011; Wang et al., 2012; Liu et al., 2013). The Schiff base cyclizes to the corresponding N-substituted glycosylamine, undergoes an irreversible Amadori rearrangement, and forms the reaction product 1-amino-1-deoxy-2-ketose (Davis et al., 1998; Gerrard, 2002; Liu et al., 2013). This reaction is the first of several parallel and sequential reactions (Singh, 1991). The Maillard reaction between a reducing fructose and a free amino group yields an irreversible Heyns rearrangement (Zerong, 2010) (**Figure 22**).

Another chemical reaction that may occur during the thermal process is lipid peroxidation, resulting in modification of amino-acid residues (Singh, 1991; Wasowicz et al., 2004). External compounds can also interfere; for example, tannins are known to precipitate proteins (Hagerman, 1992) and polyphenol compounds form soluble complexes with proteins (Siebert et al., 1996). Polyphenol oxidase can also cause enzymatic modifications (Daniel Ferreira et al., 2010).

All these chemical modifications can influence the allergenicity of proteins. There are several examples of proteins (e.g. milk and egg allergens) whose allergenicity decreases upon thermal processing, because most of the epitopes which antibodies recognize in the native protein are no longer recognized in the denatured protein (Nowak-Wegrzyn et al., 2009; Bloom et al., 2014; Leonard et al., 2016). Yet the allergenicity can also increase. For example, Maillard reactions lead to aggregation of the Ara h1 and Ara h2 proteins. The resulting aggregate is more resistant to

digestion in the gastrointestinal tract and, as a consequence, IgE binding is more efficient and stronger than for unmodified proteins (Mills et al., 2009).

The chemical modification of allergens during food processing can be analyzed by high-resolution mass spectrometry (Coward et al., 1998; Jones et al., 1998; Fay et al., 2005). In the present work, however, as the goal was to develop a multi-allergen method for detecting and quantifying allergens by mass spectrometry in processed and unprocessed foodstuffs, only peptides of native proteins, without any chemical modifications, were taken into account, so as to ensure detection of allergens in a large number of (processed and unprocessed) food products.

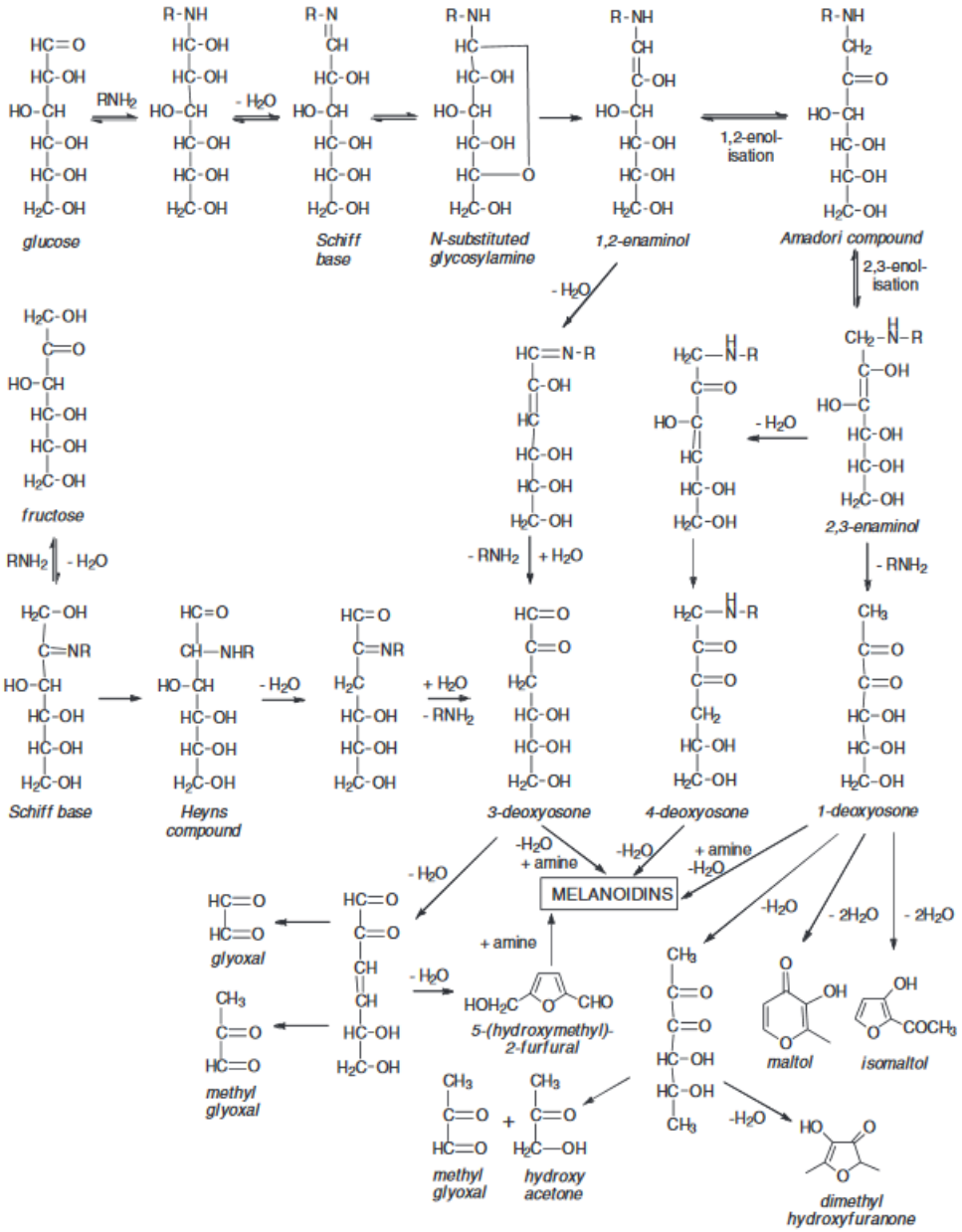


Figure 22: *The Maillard reactions, with formation of Amadori (reducing aldehyde group) and Heyns (reducing ketone group) intermediates (from* (Halford et al., 2011)*).*

VI.III Food allergen analysis: detection, quantification, and validation

This book chapter gives an overview of existing mass spectrometry methods for the detection of incurred allergens (allergens incorporated before an industrial process such as the thermal process). Also described are the different strategies for quantifying allergens (labeled proteins, peptides, standard addition…) and lastly, the validation of methods with the different criteria to be met.

Chapter 2

Food Allergen Analysis: Detection, Quantification and Validation by Mass Spectrometry

Mélanie Planque, Thierry Arnould and Nathalie Gillard

Additional information is available at the end of the chapter

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Abstract

Worldwide, food-allergy-related diseases are a significant health problem. While the food industry works on managing cross-contaminations and while clinicians deal with treatment, laboratories must develop efficient analytical methods to ensure detection of hidden allergens that can cause severe adverse reactions. Over the past few years, huge progress has been made in mass spectrometry for the analysis of allergens in incurred and processed foodstuffs, especially as regards sample preparation and enrichment (solid phase extraction, protein precipitation and ultrafiltration). These achievements make it possible to meet the Allergen Bureau's Voluntary Incidental Trace Allergen Labelling (VITAL) sensitivity criteria. The present chapter details the different steps in the development of mass spectrometry methods, from peptide selection to the validation of qualitative and quantitative methods. The chapter focuses mainly on studies performed with incurred and processed food samples to ensure the applicability of the methods to allergen detection in real food products.

Keywords: allergens, advances, detection, quantification, challenges, mass spectrometry, UHPLC-MS/MS, validation

1. Introduction

Food allergies have increased significantly, affecting between 3 and 4% of adults and at least 6% of children [1]. According to the European Academy of Allergology and Clinical Immunology (EAACI), the prevalence of food allergy has doubled over the past 10 years [2]. After an adverse reaction to a foodstuff, which may range from mild to severe (e.g.

anaphylaxis) [3, 4], allergic patients have to exclude that foodstuff from their diet. Each year in the United States, some 100 deaths are caused by anaphylaxis due to food allergy [5], the main culprits being allergens from peanut, tree nuts, fish, shellfish and milk [6]. Currently, there exist no treatments for food allergy, but clinical trials have been performed to test subcutaneous immunotherapy and oral immunotherapy used to desensitize patients [7]. The high level of adverse reactions observed in these trials has led clinicians to find safer alternative therapies, such as sublingual and epicutaneous immunotherapy. These approaches consist, respectively, in placing allergens (drops or tablets) under the tongue or in using a skin patch to induce sustained protection against anaphylaxis [8]. Although they do not treat allergic disease, they improve considerably the quality of life of highly allergic patients and constitute a real hope for them [9, 10]. The number of potentially allergenic ingredients that must appear on food labels differs in different parts of the world [11]. In Europe, regulation (EU) 1169/2011 imposes indicating the following 14 ingredients: milk, peanut, egg, soybean, fish, crustaceans, cereals containing gluten, tree nuts, celery, lupin, mustard, sesame, molluscs and sulfur dioxide [12]. This regulation fails to take into account the accidental introduction of allergens during production, transportation or storage, even though allergens introduced in this manner can trigger severe reactions [13–15]. To protect food consumers, the industry has widely used precautionary allergen labelling (PAL) (i.e. statements such as 'may contain', 'may contain traces of…') [16]. Yet, the lack of correlation between the presence of allergens and precautionary labelling has led customers to lose trust in food labels [17–20]. In a study of food product recalls over a four-year period in the European Union, the United States, Canada, Hong Kong, Australia and New Zealand, 42–90% of the recalls, depending on the country, were justified by the presence of allergens not indicated on the label [21]. Between 2007 and 2012, the Food and Drug Administration (FDA) recalled 732 products because of allergen contaminations [22] and allergic reactions are due to five foods: milk, egg, peanuts, wheat and soybean (**Figure 1**).

The distribution of these recalls in the European Union, reported in **Figure 2**, shows that the products most commonly involved in food recalls are cereals and bakery products.

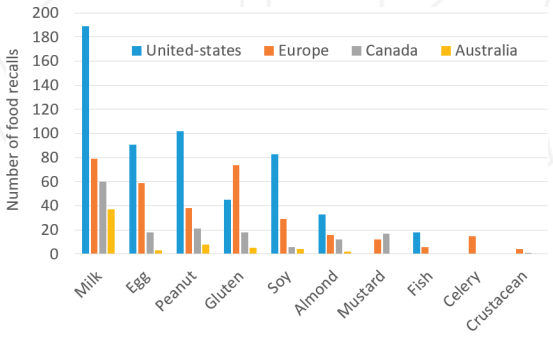

Figure 1. Number of food recalls per allergen category in the United States, Europe, Canada, and Australia between 2012 and 2015 [23–26].

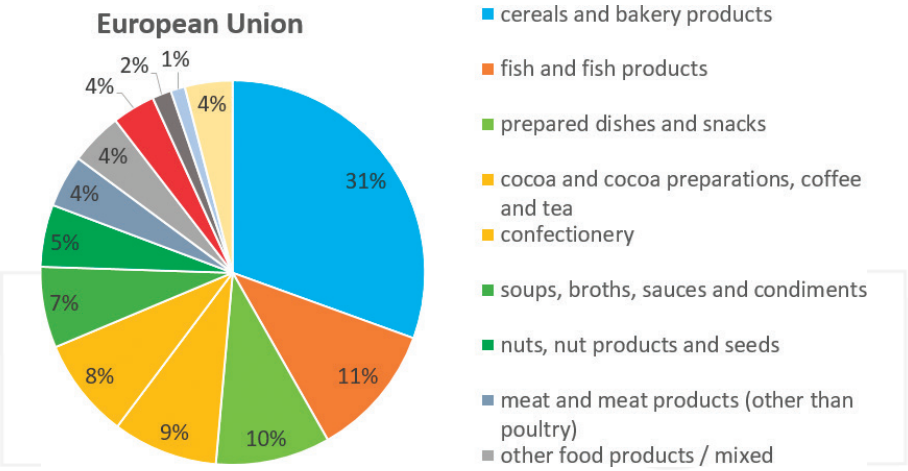

Figure 2. Percentage distribution of food allergen recalls in the European Union (according to the Rapid Alert System for Food and Feed) [24].

The widespread use of PAL can be explained by the lack of regulatory thresholds and the complexity of food allergen management through the supply chain. To counter this lack, the Voluntary Incidental Trace Allergen Labelling (VITAL) system has been developed in Australia and New Zealand to assist food producers in managing cross-contaminations during food production [27]. This system sets allergen thresholds, based on clinical studies, for the protection of 95–99% of the allergic population. Other referentials for allergen thresholds are the European Academy of Allergy and Clinical Immunology (EAACI) and the Netherlands Food and Consumer Product Safety Authority (NVWA) [28] (**Table 1**).

While the systems just mentioned have no regulatory value, food laboratories use them in evaluating method sensitivity. To obtain a concentration expressed in 'mg proteins per kilogram', a food portion size must be considered in order to compare the analytical method with VITAL thresholds (e.g. a portion size of 50 g, **Table 1**). Yet while VITAL thresholds are expressed in 'mg proteins', laboratories express their results in 'mg ingredients' [29, 30] or may refer either to soluble proteins [31, 32] or total proteins [33] per kg. To compare method performances, a conversion factor must thus be applied (e.g. 25% proteins in whole peanuts [34]). Moreover, VITAL action levels have been determined from clinical studies, mostly on the basis of the allergenicity of raw ingredients, although studies have demonstrated a major decrease in allergenicity in baked products. For example, 50–85% of allergic children are able to tolerate baked egg [35] and a study published in 2015 found 63% to tolerate 3.8 g egg-white protein in baked-egg products [36].

Nevertheless, the prevalence of baked product recalls confirms that laboratories must develop sensitive methods for detecting allergens in processed foodstuffs. The most widely used methods are based on the recognition of allergen proteins by antibodies, notably lateral flow device methods and enzyme-linked immunosorbent assays (ELISAs) [39]. DNA-based meth-

Food	Reference dose VITAL (mg of proteins) [27, 34, 37]	Reference dose |EAACI (mg of proteins) [38]	Reference dose NVWA (mg of proteins) [28]	Reference dose VITAL (mg of proteins per kg) Portion size: 50 g
Peanut	0.2	0.2	0.015	4
Cow milk	0.1	0.1	0.016	2
Egg	0.03	0.03	0.0043	0.6
Hazelnut	0.1	0.1	0.011	4
Soy	1.0	1.0	0.078	20
Wheat	1.0	1.0	0.14	20
Cashew	2.0	2.0	1.4	40
Mustard	0.05	0.05	0.022	1
Lupin	4.0	4.0	0.83	80
Sesame	0.2	0.2	0.10	4
Shrimp	10	10.0	3.7	200
Fish	/	0.1	/	/

Table 1. VITAL (http://allergenbureau.net/vital/), EAACI (http://www.eaaci.org/) and NVWA (https://www.nvwa.nl/) reference doses for different food allergens.

ods such as those exploiting the real-time polymerase chain reaction (PCR) [40] are also used to detect the presence of allergens. Currently, mass spectrometry is becoming an alternative to these methods, as heat-processing induces protein denaturation and structural modifications that might result in non-recognition of the target protein by conformational antibodies and thus in the case of ELISAs, lead to false negatives or at least major underestimation of allergen content [41–44]. Mass spectrometry has the advantage of permitting simultaneous analyses for several allergens in food, including processed food products, with high sensitivity and specificity.

This chapter highlights the important improvements made over the last 10 years in mass spectrometry applied to the development of allergen detection methods. It covers and discusses the mass spectrometry methods currently used to detect and quantify allergens in processed food products, including their validation.

2. Detecting food allergens

2.1. Selecting marker peptides

Food allergens (except sulfites) are proteins that need to be digested by enzymes (trypsin and chymotrypsin) so as to generate peptides suitable for routine mass spectrometry analysis. Identification and selection of robust peptides are generally done first on digested raw ingredients before analysis of digested processed ingredients in food matrices. This section

summarizes two approaches commonly used to select marker peptides (the instrumental approach and the *in silico* approach) and the specificity and sensitivity critera used.

2.1.1. Peptide selection

2.1.1.1. Instrumental peptide selection

The first approach is to identify abundant marker peptides by high resolution mass spectrometry (HRMS). Downstream from allergen analysis by HRMS, the generated data are transferred into an algorithm for assigning peptides to MS/MS spectra (MASCOT, X!Tandem, SEQUEST) [45]. For example, Sealey-Voyksner et al. analysed 12 tree nuts and peanut-raw and roasted (176.7°C, 30 min) by time of flight (q-TOF) (Agilent 6530) spectrometry and selected two abundant peptides per tree nut and four for peanut [46]. In a previous study, ice cream spiked with peanuts was analysed by q-TOF (Waters Micromass II) to identify peptides of the Ara h1 allergen [47]. In a 2012 study, Cucu et al. identified several soybean marker peptides by matrix-assisted laser desorption ionization (MALDI-TOF/MS) [48]. The main advantage of this approach is that global peptide and protein profiles can be analysed for the different samples.

2.1.1.2. In silico peptide selection

Another strategy for selecting marker peptides is to retrieve target protein sequences from a database, e.g. Uniprot (http://www.uniprot.org/), and to perform an *in silico* digestion with an open access software, e.g. Skyline or MRMaid [49, 50] (**Figure 3**).

In silico digestion with multiple reaction monitoring (MRM) involves generating a list of criteria that must be applied or set by the user as regards peptides, transitions and MS/MS

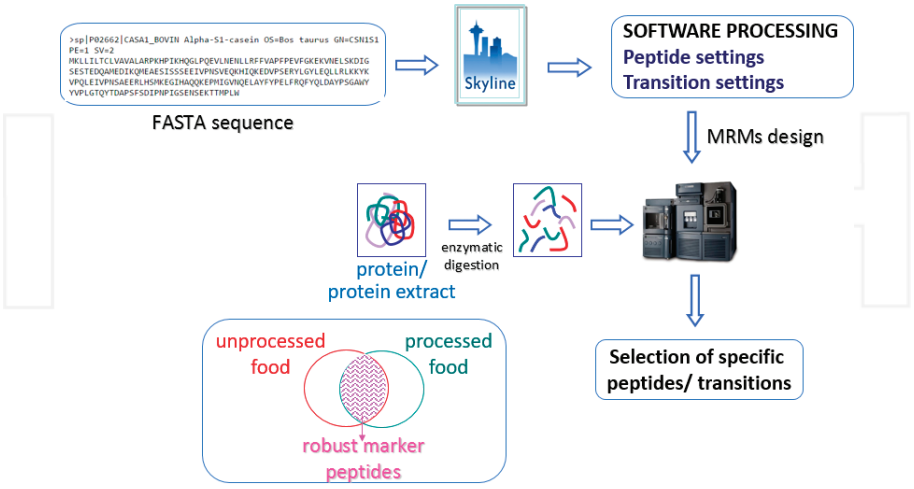

Figure 3. *In silico* peptide selection with the Uniprot database and Skyline software.

parameters (e.g. peptide length, charge states, fragmentation and enzyme). Then raw ingredients or incurred matrices can be analysed by ultra-high performance liquid chromatography coupled to tandem mass spectrometry (UHPLC-MS/MS). This approach allows identification of abundant peptides. It was used by Rogniaux et al. for the analysis of wheat varieties: several gluten peptides were identified with a linear ion trap quadrupole mass filter in tandem with an orbitrap (Thermo Fisher Scientific) [51].

An *in silico* approach also requires a complete database with available protein sequences. Uniprot inventories, however, can be too large (e.g. >145,000 proteins for the wheat species-*Triticum aestivum*), making it necessary to first select target proteins from the literature. Use of a routine UHPLC-MS/MS instrument is the main advantage of the *in silico* approach for laboratories unwilling to invest in a high-resolution mass spectrometer.

2.1.2. Specificity

BLAST: After this selection, blasting must be performed to guarantee the specificity of marker peptides. This step is mandatory but not always included in method development. In one study, for example, Hoofnagle et al. selected five peptides for the detection of β-casein in cookies: EMPFPK (6AA), VLPVPQK (7AA), AVPYPQR (7AA), GPFPIIV (7AA) and DMPIQAFLLYQEPVLGPVR (19AA) [52]. Only one of these peptides could be blasted, and this peptide is 100% homologous to goat, zebu, buffalo, yak and sheep β-casein (Uniprot). In proteomics, peptide blasting should be systematic, even though the international trade frequently introduces new food products and although some proteins can still be missing in the different databases.

The **specificity of selected fragments** is also paramount. To improve specificity, the mass-to-charge ratio (m/z) of the precursor should be lower than the m/z of the fragments. Too-small fragments should be avoided. At least, fragments of 1 to 2 amino acids (b1, b2, y1, y2) should be excluded, which is not always the case in published methods [53, 54].

Blanks: Matrices without allergens must also be analysed to ensure the specificity of the selected transitions of the target peptides. As databases do not cover all possible proteins and as new food products enter the food chain regularly, this experimental testing is crucial to proving method specificity.

2.1.3. Identifying marker peptides in incurred foodstuffs

The advantage of using mass spectrometry is detection of allergens in industrial food products. For such applications, only target peptides and proteins that will be detected in incurred and processed matrices, such as those listed in **Table 2**, need to be retained in the analytical methods. Some peptides are common to the majority of published methods: FFVAPFPEVFGK and YLGYLEQLLR (Casein αS1), and GGLEPINFQTAADQAR (ovalbumin), among others. Target peptides detected after different extraction and purification steps in several types of matrices constitute potential marker peptides for the detection of allergens in a wide variety of foodstuffs.

Authors	Matrix	Allergen	Protein	Peptide	Fragments
Heick et al. [53]	Bread (60 min, 200°C)	Milk	αS1-casein	YLGYLEQLLR	b2, y8
				FFVAPFPEVFGK	y8, y9
			αS2-casein	NAVPITPTLNR	b2, y8
				FALPQYLK	a1, y5
		Egg	Ovalbumin	HIATNAVLFFGR	a2, y10
				YPILPEYLQCVK	y6, y8
				DILNQITKPNDVYSFSLASR	a2, y8
				ELINSWVESQTNGIIR	y9, y10
		Soy	Glycinin	NLQGENEGEDKGAIVTVK	a2, b3
				VFDGELQEGR	a2, y8
				SQSDNFEYVSFK	y3, y10
				EAFGVNMQIVR	y6, y8
		Peanut	Ara h1	DLAFPGSGEQVEK	a3, y9
				GTGNLELVAVR	y5, y6
			Ara h3/4	RPFYSNAPQEIFIQQGR	y6, b7
				WLGLSAEYGNLYR	a2, y11
		Hazelnut	11S globulin	ADIYTEQVGR	y6, y7
				INTVNSNTLPVLR	y4, y9
				QGQVLTIPQNFAVAK	y8, y10
				ALPDDVLANAFQISR	y8, y9
		Walnut	Jug r1	DLPNECGISSQR	y4, y10
				QCCQQLSQMDEQCQCEGLR	y3, y10
				GEEMEEMVQSAR	y7, y8
		Almond	Prunin	GNLDFVQPPR	y3, y7
				GVLGAVFSGCPETFEESQQSSQQGR	y6, y7
				ALPDEVLANAYQISR	y8, y9
				NGLHLPSYSNAPQLIYIVQGR	y6, b11
Pilolli et al. 2016 [56]	Cookie (200°C, 12 min)	Milk	αS1-casein	FFVAPFPEVFGK	y8, y9, y10
				YLGYLEQLLR	y5, y6, y8
		Egg	Ovalbumin	GGLEPINFQTAADQAR	y7, y10, y12
				YPILPEYLQCVK	b4, y8, y9
		Peanut	Conarachin	VLLEENAGGEQEER	y7, y8, y12
				EGEQEWGTPGSEVR	y6, y8, y9

Authors	Matrix	Allergen	Protein	Peptide	Fragments
		Soy	Glycinin G1-G2	SQSDNFEYVSFK	y3, y10
				FYLAGNQEQEFLK	y9, y10, y11
		Hazelnut	11S globulin-like protein	ADIYTEQVGR	y6, y7
				ALPDDVLANAFQISR	y7, y8, y13
Lamberti et al. [57]	Cookie (180°C, 10 min)	Milk	αS1-casein	YLGYLEQLLR	y8, y9, y10
				FFVAPFPEVFGK	y8, y9, y10
				HQGLPQEVLNENLLR	y11, y12
Pedreschi et al. [58]	Cookie (180°C, 16 min)	Peanut	Ara h1	VLLEENAGGEQEER	y9, y8, y7, y6, y4, y2
				DLAFPGSGEQVEK	y10, y9, y8, b4, b3, b2
			Ara h2	CCNELNEFENNQR	y8, y6, y5, y4
				NLPQQCGLR	y7, y6, y5, a2
				CDLEVESGGR	y8, y6, y5, y4
				CMCEALQQIMENQSDR	y14, y11, y10, y8, y7, y6, y5, b2
			Ara h3	LNAQRPDNR	ymax, y8, y7, y5, b2
				SPDIYNPQAGSLK	ymax, y12, y9, y8, y7, y5, b3
				AHVQVVDSNGNR	b7, y6, b5
Huschek et al. [59]	Cookie (190°C, 13 min)	Soy	Gly m6	VFDGELQEGR	903.6/ 489.2/ 788.5
				LSAEFGLR	432.3/ 779.4/ 579.3
				LNALKPDNR	742.4/ 629.3/ 501.2
		Sesame	Ses i6	ISGAQPSLR	472.3/ 728.4/ 671.4
				AFYLAGGVPR	556.3/ 485.3/ 669.4
				SPLAGYTSVIR	795.4/ 866.5/ 575.4
		Lupine	β-conglutin	LLGFGINADENQR	846.4/661.3/ 797.4
				NTLEATFNTR	951.5/838.4/ 709.4
				NPYHFSSQR	761.4/ 624.3/ 477.2

Table 2a. List of target marker peptides used to detect several allergens in bread and cookies [55–59].

2.2. Developing mass spectrometry methods

After selection of marker peptides, the developed method must be able to detect traces of the allergen proteins in the 'mg allergen proteins per kg food product' range. The real chal-

Authors	Matrix	Allergen	Protein	Peptide	Fragments
Planque et al. [33, 60]	Cookie (180°C—18 min), sauce (95°C, 45 min), ice cream and chocolate	Milk	αS1-Casein	FFVAPFPEVFGK	y6, y8, y9
				HQGLPQEVLNENLLR	b4, y6, y7
				YLGYLEQLLR	y5, y6, y7
			αS2-casein	NAVPITPTLNR	b3, y8, y8
			β-lactoglobulin	VYVEELKPTPEGDLEILLQK	y11, y14, y16
				VLVLDTDYK	y5, y6, y7
				LSFNPTQLEEQCHI	y7, y10, y10
		Egg	Ovalbumin	GGLEPINFQTAADQAR	y10, y12, y12
				LTEWTSSNVM EER	y7, y8, y9
				ISQAVHAAHAEINEAGR	y9, y10, y11
			Vitellogenin	EALQPIHDLADEAISR	y6, y7, y12
				NIPFAEYPTYK	y4, y9, y9
				NIGELGVEK	y5, y6, y7
				YLLDLLPAAASHR	y7, y7, y11
			Apovitellenin	NFLINETAR	y5, y6, y7
		Peanut	Cupin	NTLEAAFNAEFNEIR	y7, y8, y9
				RPFYSNAPQEIFIQQGR	b7, y6, y10
				FNLAGNHEQEFLR	y5, y9, y10
				TANELNLLILR	y6, y7, y8
		Soy	Glycinin	ISTLNSLTLPALR	y7, y8, y9
				EAFGVNMQIVR	y5, y6, y7
				ELINLATMCR	y5, y6, y8
				LITLAIPVNKPGR	y7, y9, y11
Parker et al. [61]	Muffin (177°C, 48 min)	Egg	Lysozyme	FESNFNTQATNR	Not provided
				NTDGSTDYGILQINSR	
			Ovalbumin	ELINSWVESQTNGIIR	
				GGLEPINFQTAADQAR	
				HIATNAVLFFGR	
		Milk	αS1-casein	FFVAPFPEVFGK	
				HQGLPQEVLNENLLR	
				YLGYLEQLLR	
			β-lactoglobulin	LSFNPTQLEEQCHI	
				TPEVDDEALEK	
				VLVLDTDYK	

Authors	Matrix	Allergen	Protein	Peptide	Fragments
		Peanut	Ara h1	GTGNLELVAVR	
				NNPFYFPSR	
			Ara h2	CCNELNEFENNQR	
				CMCEALQQIMENQSDR	
				NLPQQCGLR	
			Ara h3	FNLAGNHEQEFLR	
				SPDIYNPQAGSLK	
				WLGLSAEYGNLYR	
Gomaa et al. 2014 [62]	Cookie (177°C, 12 min)	Milk	αS1-casein	HQGLPQEVLNENLLR	best transitions not selected
			αS2-casein	NAVPITPTLNR	
				LNFLK	
				ALNEINQFYQK	
			κ-casein	YIPIQYVLSR	
		Soy	Glycinin G1 precursor	HNIGQTSSPDIYNPQAGSVTTATSLDFPALSWLR	
				TNDTPMIGTLAGANSLLNALPEEVIQHTFNLK	
				VLIVPQNFVVAAR	
				HQQEEENEGGSILSGFTLEFLEHAFSVDK	
				EGDLIAVP…DQMPR	
			Glycinin G2 precursor	TNDRPSIGNLAGANSLLNALPEEVIQHTFNLK	
				QNIGQNSSPDIYNPQAGSITTATSLDFPALWLLK	
			Beta conglycinin alpha chain precursor	DLDIFLSIVDMNEGALLLPHFNSK	
				AIVILVINEGDANIELVGLK	
		Wheat	Alpha amylase trypsin inhibitor	YFIALPVPSQPVDPR	
				LLVAPGQCNLATIHNVR	
				LTAASITAVCR	
				LPIVVDASGDGAYVCK	
				SGNVGESGLIDLPGCPR	
				EMQWDFVR	
				DYVLQQTCGTFTPGSK	

Table 2b. List of target marker peptides used to detect several allergens in sauce, ice cream, chocolate, cookies and muffins [60–62].

lenge for laboratories is to achieve this sensitivity with processed foodstuffs. To reach this sensitivity, two factors must be considered: instrument sensitivity and optimization of sample preparation. The different strategies used to evaluate sensitivity are described below.

Instrument sensitivity: No comparison of the sensitivities of different instruments with the same peptide extract has yet been published for allergen analysis, although the sensitivity of the instrument is crucial to the sensitivity of the method, as in the case of other contaminants. One should bear in mind, however, that the most sensitive research-dedicated instrument might not be the best choice for routine analysis (automated injection and short analytical run).

Extraction and purification of proteins: The ideal sample preparation protocol should allow extraction of 100% of the target compounds, the final extract used for MS analysis being as pure as possible. Yet, the preparation of samples for food allergen analysis is difficult, because it should be applicable to a very broad range of food matrices and because the extractability of proteins might be altered in a processed food [63]. In addition, several modifications can occur, e.g. asparagine deamination, the Maillard reaction and several reactions of lysine. Such modifications cause a mass shift of tryptic peptides, resulting in non-recognition of several peptides by mass spectrometry [64–66]. To improve protein extraction, different parameters can be optimized: the composition of extraction buffers, the temperature, the sample-to-buffer ratio and the presence of detergents. Furthermore, the purification step is as important as extraction in order to concentrate proteins in and eliminate interferences from the supernatant. Purification usually involves solid phase extraction (SPE), protein precipitation, ultrafiltration and size exclusion chromatography (SEC), among others. Optimizing extraction and purification is a key step in developing sensitive methods for the detection of allergens by mass spectrometry (**Table 3**).

Determining the sensitivity: The sensitivity of food allergen analysis can be determined on spiked samples (obtained by incorporating extracted proteins into a matrix after processing), fortified samples (obtained by incorporating raw ingredients into a matrix after processing) or processed samples (obtained by incorporating raw ingredients into a matrix before processing). For spiked and fortified samples ('non-processed samples'), examples of the limit of quantification (LOQ) reached are 0.1 mg milk protein, 0.3 mg egg protein and 2 mg soy protein per kg cookies [67] and 0.1–1.3 mg tree nuts per kg biscuit [68]. Although these studies demonstrate the sensitivity of mass spectrometry, the real challenge is to reach this sensitivity in thermally processed samples. Important improvements have been made over the last 5 years in the detection of allergens in processed samples. Recently, developed methods allow reaching an LOQ near or below the VITAL threshold (**Table 1**), e.g. 0.5 mg for milk protein, 3.4 mg egg protein, 5 mg soy protein and 2.5 mg peanut protein per kg incurred cookie (180°C, 18 min, with SPE purification) [60]. In another study, the LOQs achieved were 30 mg egg (13.8 mg proteins), 20 mg milk (7.2 mg proteins), 19 mg soy (6.8 mg proteins), 20 mg hazelnut (3 mg proteins) and 40 mg peanut (10 mg proteins) per kg incurred cookie (200°C, 12 min, with SEC purification) [56].

As described above, the sensitivity reached for processed samples is lower than that obtained with spiked or fortified samples. The same applies to ELISAs, which can show up to 100-fold lower sensitivity when applied to processed food than when applied to raw food, as demonstrated by the poor performance of several ELISAs for egg detection in cookies after processing. In 2010, Dumont et al. showed that one ELISA kit was not even able to detect 1000 mg egg powder per kg baked cookie, and four others strongly underestimated the amount of egg in

Authors	Allergen	Matrix	Extraction	Purification	Digestion	Instrument	Sensitivity
Heick et al. (2010) [55]	Milk, egg, soy, peanut, hazelnut, walnut, almond	Bread (200°C, 60 min)	2 g/20 ml	Ultrafiltration (Amilcon Ultra 15 mL, 5 kDa molecular weight cut-off) (Millipore)	Dilution: 1 mg of proteins by ml with NH4HCO3 (100 mM)	LC: 1200 HPLC (Agilent)	LOD (S/N>3)
			Buffer: TRIS-HCl pH 8.2		Aliquot: 100 µl	Column: Xbridge C18 3.5 µm (2.1×150 mm) (Waters)	5 mg of soluble milk proteins by kg
			Agitation: 60°C for 3h		Reduction: 50 µl DTT (200 mM), 45 min	MS: API 4000QTrap (MDS Sciex)	42 mg of soluble egg proteins by kg
					Alkylation: 40 µl IA (1 M), 45 min in the dark		24 mg of soluble soy proteins by kg
					20 µl DTT (200 mM) + 50 µl NH4HCO3 (100 mM)		11 mg of soluble peanut proteins by kg
					Digestion: 10 µl trypsin (1 µg/µl) 12 h - 37°C		5 mg of soluble hazelnut proteins by kg
					2 µl formic acid		70 mg of soluble walnut proteins by kg
							3 mg of soluble almond proteins by kg
Pilolli et al. [56]	milk, egg,soy,peanut, hazelnut	Cookie (200°C, 12 min)	2.5 g/50 ml	1.2 µm acetate cellulose membrane, Size exclusion column (SEC) (G25 Sephadex column)	Elution SEC: 3.5 ml NH4HCO3 (50 mM)	LC: -	LOD (S/N>3)
			Buffer: 20 mM TRIS-HCl pH 8.2		Aliquot: 300 µl	Column: Aeris Peptide XB-C18 (150 × 2.1 mm) (Phenomenex)	7 mg of milk by kg
					Protein denaturation: 15 min at 95°C	MS: Dual pressure Linear Ion Trap Spectrometer Velos Pro (Thermo Fisher Scientic)	9 mg of egg by kg
			Ultrasound: 30 min		Reduction: 15 µl of 50 mM DTT 30 min at 60°C		6 mg of soy by kg
					Alkylation: 30 µl of 100 mM IAA 30 min in the dark at room temperature		13 mg of peanut by kg

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Authors	Allergen	Matrix	Extraction	Purification	Digestion	Instrument	Sensitivity
					Digestion:4 µl trypsin (1 µg/µl) 14h		7 mg of hazelnut by kg
					Acidification: 1M HCl		
					Filtration: 0.2 µm		
Lamberti et al. [57]	Milk	Cookie (180°C, 10 min)	10 mg /200 µl	Protein precipitation methanol/ chloroform	Pellet + 50 µl of 0.025M NH4HCO3 pH 8.0 RT	LC: HP 1100 HPLC (Agilent)	LOD: 1.3 mg of milk proteins per kg
			Buffer: NH4HCO3 / (NH4)2CO3 +1 % SDS buffer, pH 8.2		3 µl of trypisn (75 ng/µl) 37°C, 90 min	Column: ACE C18 300A (250 mm × 1 mm)	LOQ: 4 mg of milk proteins per kg
			Agitation: 20 min, 60°C		5 µl of 5% formic acid	MS: XCT-Plus Ion trap mass spectrometer (Agilent)	
Pedreschi et al. [58]	Peanut	Cookie (180°C, 16 min)	Buffer: 20 mM TRIS - 150 mM NaCl, pH 7.4	GE Healthcare kit	50 µg of protein / 50 µl of Rapigest in a 50 mM ammonium bicarbonate buffer	LC: nano Acquity UPLC (Waters)	>10 mg of peanut per kg
			Ultrasound: 4°C, 20 min	Cut-off filtration 3000 MWCO	2.5 µl of 50 mM DTT 30 min, 60°C	Column: nano Acquity BEH130 C18 1.7 µm (75 µm × 100 mm)	
					5 µl 100 mM IAA 30 min-dark	Column: nano Acquity UPLC Trap SymC18 5 µm (180 µm × 20 mm)	
					1 µl of 1µg/µm of trypsin 5h-37°C	MS: Q-Tof Ultima Global (Waters)	

Table 3a. Mass-spectrometry-based methods (extraction, purification, digestion, and analysis) for detecting allergens in processed food products [55–58].

Author	Allergens	Matrices	Extraction	Purification	Digestion	Instrument	Sensitivity
Huschek et al. [59]	Soy, sesame, lupin	Wheat, cookie (190°C, 13 min), bread (220°C, 30 min)	1 g	SPE cardridge (LiChrolut RP-18 Merck Millipore)	Alkylation: IAA 20 min at 50°C	LC: Nexera XR UHPLC (Shimadzu)	LOQ (S/N > 10)
			Buffer: 100 mM NH4HCO3, 4M urea 5 mM DTT pH 8.2		Digestion: Trypsin formic acid 2%	Column: Aeris Peptide XB-C18 (100 × 2.1 mm, 1.7 µm) (Phenomenex)	10–20 mg of soy per kg 10–50 mg of sesame per kg
			Agitation 30 min RT			MS: Qtrap 5500 MS/MS (Sciex)	10–50 mg of lupine per kg
Planque et al. [33, 60]	Milk, egg, soy, peanut	Tomato sauce (95°C, 45 min), cookie (180°C, 18 min), ice cream, chocolate	2 g / 20 ml	Sep-Pack tC18 6cc (Waters)	10 ml extract + 10 ml NH4HCO3 (200 mM)	LC: UPLC Acquity (Waters)	LOQ (S/N > 10)
			Buffer: 200 mM TRIS-HCl pH 9.2, 2M urea		Reduction: 1 ml DTT (400 mM), 45 min	Column: BEH130 (2.1 × 150 mm) (Waters)	0.5 mg of milk proteins by kg
					Alkylation: 2 ml IAA (500 mM), 45 min in the dark	MS: Xevo TQS (Waters)	3.4 mg of egg proteins by kg
			Agitation:30 min Ultrasound: 15 min		Digestion: Ratio protein:trypsin 1:20 16 h, 37°C		5 mg of soy proteins by kg
					300 µl formic acid 20%		2.5 mg of peanut proteins by kg
Parker et al. [61]	Milk, egg, peanut	Muffin (177°C, 48 min)	Buffer: 2 M urea, 50 mM TRIS Ph 8.0, 25 mM DTT	Microcentrifuge tubes	Filter-aided sample preparation (FASP) sample cocentration and digestion protocol	LC: nano Acquity UPLC (Waters)	/
				Amicon Ultra 0.5 ml	Reduction: 10 mM DTT	Column: nano Acquity BEH130 C18 1.7 µm (100 µm × 100 mm)	
			vortex: 5 min at 1400 rpm	Utracel-10 membrane	Alkylation:25 mM IAA	MS:6500 QTRAP (Sciex)	
			Ultrasound: 10 min at 4°C		Digestion: Ratio protein:trypsin 1:100 16 h -37°C		
					0.1% trifluoroacetic acid and 2% acetonitrile		

Author	Allergens	Matrices	Extraction	Purification	Digestion	Instrument	Sensitivity
Gomaa et al. [62]	Milk, soy, wheat	Cookie (177°C, 12 min)	1 g/9 ml	OMIX C18/ tip (Varian)	Protein extract: 100 μl at 2 mg/ml	LC: nano Acquity UPLC (Waters)	10 mg of casein per kg
			Buffer: 50 mM NH4HCO3		Reduction: 1μl of DTT (0.5 M) 56°C, 20 min	Column: nano Acquity BEH130 C18 1.7 μm (75 μm × 100 mm)	10 mg of soy proteins per kg
					Alkylation: 2.7 μl IAA (0.55 M, 15 min)	nano Acquity UPLC Trap SymC18 5 μm (180 μm × 20 mm)	100 mg of gluten per kg
					Digestion: ratio enzyme:subtrate 1:20 3h-37°C	MS: Q-Tof Synapt MS (Waters)	
					5 μl trifluoroacetic acid (2.5%)		

Table 3b. Mass-spectrometry-based methods (extraction, purification, digestion, and analysis) for detecting allergens in processed chocolate, sauce, ice cream, muffins and cookies [59–62].

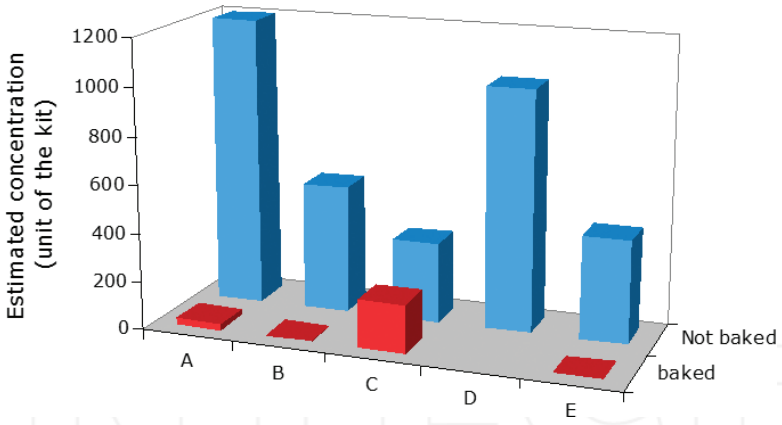

Figure 4. Analytical results for 1000 mg spray-dried whole egg powder (National Institute of Standards and Technology RM 8445) per kg incurred cookies, obtained with the different enzyme-linked immunosorbent assay test kits for egg detection (A–E) (from Ref. [69]).

the samples (**Figure 4** of Ref. [69]). While mass spectrometry and ELISAs show comparable sensitivities when applied to unbaked products, mass spectrometry seems to be the method of choice for the analysis of allergens in baked food products.

3. Quantifying food allergens

Detecting hidden allergens in food products is essential to protecting the food-allergic population. For full transparency of allergen labelling, laboratories should also be able to quantify allergens in order to help food manufacturers manage cross-contamination during food production [70]. However, significant signal suppressions have been observed in various food matrices, and the level of suppression depends on the matrix considered. In one study, for example, high-protein-content food products showed greater suppression of the peptide signal than ones with a low protein content: the determined LOQ values were 20 mg skim milk powder per kg for high-protein foods and 5 mg skim milk powder per kg for low-protein foods [71]. The food protein content is not the only parameter to be considered in relation to suppression of the peptide signal obtained by mass spectrometry: factors such as the type of process, the fat content and the presence of tannins also have an important influence on food allergen detection and must be taken into account.

While detecting allergens in various food products is difficult, quantifying them is even worse. In recent years, mass spectrometry techniques have been used for quantitation in proteomic analysis. Two approaches have emerged as the most relevant for food allergen quantification: label-free quantification and the use of stable-isotope-labelled peptides or proteins [70, 72, 73]. The two strategies are compared in **Table 4** (target peptides, internal standards and calibration curves) and discussed in relation to the AOAC guideline 2016.002 method performance requirements for the quantification of allergens in food products, specifying a recovery between 60 and 120% and intra-day and inter-day coefficients of variation lower than 20 and 30%, respectively [74] (**Table 5**).

Authors	Matrix	Allergen	Protein	Peptide	Mass spectrometer	Internal standard	Calibration curve
Careri et al. [76]	Rice crispy/ chocolate snacks	Peanut	Ara h2	CCNELNEFENNQR	Q-TOF Micro (Waters)	No internal standard	Rice crispy/ chocolate snacks were spiked with peanut proteins
				CMCEALQQIMENQSDR			
			Ara h3/4	AHVQVVDSNGDR			
				SPDIYNPQAGSLK			
Monaci et al. [75]	Fruit juices	Milk	α-lactalbumin	/	Ultima triple quadrupole mass spectrometer (Waters)	No internal standard	Fruit juices were spiked with milk proteins
			β-lactoglobulin A				
			β-lactoglobulin B				
Korte et al. [88]	Bread matrix, ice cream, chocolate, muesli with fruit and berry	Almond	Pru du 6.0101	GNLDFVQPPR	QTRAP 6500 (Sciex)	No internal standard	Matrices were spiked with allergen proteins
			Pru du 6.0201	VQGQLDFVSPFSR			
				ALPDEVLQNAFR			
		Cashew	Ana o2	ADIYTPEVGR			
				EGQMLVVPQNFAVVK			
				LTTLNSLNLPILK			
		Hazelnut	Cor a 9	LNALEPTNR			
				VQVVDDNGNTVFDDELR			
				QGQVLTIPQNFAVAK			
		Peanut	Ara h3	FNLAGNHEQEFLR			
				WLGLSAEYGNLYR			
				TANDLNLLILR			

Authors	Matrix	Allergen	Protein	Peptide	Mass spectrometer	Internal standard	Calibration curve
		Pistachio	Pis v 5	AMISPLAGSTSVLR			
				ITSLNSLNLPILK			
				GFESEEESEYER			
		Walnut	Jug r 2	FFDQQEQR			
				ATLTLVSQETR			
			Jug r4	ALPEEVLATAFQIPR			
Mattarozzi et al. [77]	Pasta, biscuit	Lupin	β-conglutin	IVEFQSKPNTLILPK	LTQ XL linear ion trap (Thermo)	No internal standard	Pasta and biscuits were fortified with lupin proteins
Zhang et al. [78]	Infant formulas and whey proteins	Milk	α-lactalbumin	VGINYWLAHK	Xevo TQ triple quadrupole (Waters)	KILDKVGINNYWLAHKALCSE	Matrices were spiked with synthetic peptide VGINYWLAHK
Posada-Ayala et al. [79]	Sauces and salty biscuit	Mustard	Sin a1	ACQQWLHK	6460 triple quadrupole (Agilent technologies)	Purified protein Sin a 1	Standard addition of mustard in sauces and salty biscuits
				IYQTATHLPK			
				EFQQAQHLR			

Table 4a. Quantification of food allergens in different food products by mass spectrometry using label-free quantification with an (1) external calibration curve [75–79, 88], (2) unlabelled modified synthetic peptide [78], and (3) standard addition [79].

Authors	Matrix	Allergen	Protein	Peptide	Mass spectrometer	Internal standard	Calibration curve
Newsome and Scholl [82]	Cookie (180°C, 16 min)	Milk	α-s1 casein	HQGLPQEVLNENLLR	Hybrid triple-quadrupole 4000 QTRAP (AB Sciex)	HQGLPQEVLNENLLR[13C, 15N]	Cookies were spiked with isotope labelled peptides
				YLGYLEQLLR		YLGYLEQLLR[13C6, 15N]	
				FFVAPFPEVFGK		FFVAPFPEVF[13C6, 15N]GK	
						15N-α-s1 casein	
Parker et al. [61]	Cereal bar (177°C, 30 min) Muffin (177°C, 48 min)	Egg	Lysozyme	NTDGSTDYGILQINSR	6500 Qtrap (AB Sciex)	Heavy isotope [13C, 15N] labelled peptides / labelled amino acid: R or K	Cereal and muffin were spiked with isotope labelled peptides
			Ovalbumin	GGLEPINFQTAADQAR			
		Milk	α-s1 casein	YLGYLEQLLR			
			β-lactoglobulin	LSFNPTQLEEQCHI			
		Peanut	Ara h1	NNPFYFPSR			
			Ara h2	NLPQQCGLR			
			Ara h3	SPDIYNPQAGSLK			
Huschek et al. [59]	Wheat, cookies (190°C, 13 min), soft bread (220°C-	Soy	Gly m6	VFDGELQEGR	QTRAP 5500 (Sciex)	VFDGELQEGR[13C6, 15N4]	Wheat and cookies were spiked with allergen proteins
		Sesame	Ses i6	ISGAQPSLR		ISGAQPSLR[13C6, 15N4]	
		Lupin	β-conglutin	LLGFGINADENQR		LLGFGINADENQR[13C6, 15N4]	
Lutter et al. (2011) [71]	Baby food soy-based formula, infant cereals, breakfast cereals, rince water	Milk	β-casein	AVPYPQR	6460 triple quadrupole (Agilent technologies)	AVPYPQR [13C6, 15N4]	0.1% formic acid were spiked with proteins
			α-s2 casein	ALNEINQFYQK		ALNEINQFYQK[13C6, 15N2]	
			α-s2 casein	FALPQYLK		FALPQYLK[13C6, 15N2]	
			κ-casein	YIPIQYVLSR		YIPIQYVLSR[13C6, 15N2]	
			β-lactoglobulin	TPEVDDEALEK		TPEVDDEALEK[13C6, 15N2]	
				VLVLDTDYK		VLVLDTDYK[13C6, 15N4]	
Monaci et al. [75]	White wine	Milk	α-s1 casein	FFVAPFPEVFGK	Extractive ESI Orbitrap (Thermo Electron)	FFV[15N]APFPEV[15N]FGK	White wine were spiked with milk and egg proteins
		Egg	Ovalbumin	LTEWTSSNVMEER		LTEWTSSNV[15N]MEER	

Authors	Matrix	Allergen	Protein	Peptide	Mass spectrometer	Internal standard	Calibration curve
Yi-Shun et al. (2017) [84]	Beer, wine, chips, flour, cookies…	Gluten	α-glyadin	LWQIPEQSR	6490 triple quad (Agilent)	LWQIPEQSR[13C, 15N]	Matrices were spiked with gluten proteins
			γ-Hordein	QQCCQQLANINEQSR		QQCCQQLANINEQSR [13C, 15N]	
Ippoushi et al. [89]	Sweet cherry fruit	Cherry	Pru av2	TGCCAMSTDASGK	Xevo TQD Zspray ion source (Waters)	TGCCAMSTDASGK[13C6,15N2]	Sweet cherry fruit were spiekd with isotope labelled peptides
Rahman et al. (2012) [90]	/	Shrimp	Tropomyosine	SEEEVFGLQK	Micro mass Quattro Ultima (Waters)	SEEEV[D8]VFGLQK	Solvent were spiked with shrimp proteins
			Arginine kinase	QQLVDDHFLFVSGDR		QQLV[D8]VDDHFLFV[D8]SGDR	
Chen et al. (2014) [83]	Baked food (170°C, 25 min)	Milk	β-casein	VLPVPQR	TOF-MS Synapt G2 HDMS (Waters)	VL[13C6, 15N]PV[13C5, 15N]PQK (IS1)	Solvent were spiked with milk proteins
						QSVLSLSQSKVL[13C6, 15N]PV[13C5, 15N]PQKAVPYPQRQ (IS2)	
						Human β-casein (IS3)	

Table 4b. Quantification of food allergens in different food products by mass spectrometry using stable isotope labelling quantification with an (1) isotope-labelled protein [82], (2) isotope-labelled peptide [59, 61, 71, 75, 84, 89, 90] or (3) long isotope-labelled peptide [83].

Parameter	Target allergen			
	Whole egg	**Milk**	**Peanut**	**Hazelnut**
Analytical range, ppm	10–1000	10–1000	10–1000	10–1000
MLQ, ppm	≤5	≤10	≤10	≤10
MDL, ppm	≤1.65	≤3	≤3	≤3
Recovery %	60–120	60–120	60–120	60–120
RSD_r %	≤20	≤20	≤20	≤20
RSD_R %	≤30	≤30	≤30	≤30

Reported as ppm of the target allergen in food commodity i.e. 25 ppm of 'whole egg' in cookies.

Table 5. Method performance requirements from the AOAC guideline SMPR 2016.002 for egg, milk, peanut and hazelnut allergens in terms of analytical range, method quantification limit, recovery and intra-day and inter-day coefficients of variation (table from Paez et al. [74]).

3.1. Label-free quantification

The label-free quantification strategy is based on comparing the peptide signal intensities of different samples (**Table 4a**). Three label-free quantification possibilities are described below.

External calibration: Monaci et al. used this approach to quantify milk proteins in fruit juice. Using a calibration curve obtained by spiking fruit juice with extracted milk proteins, they found recoveries between 68 and 79% [75]. This strategy was also used to quantify peanut proteins in rice crispy/chocolate snacks [76]. A significant suppression effect, ranging from 30 to 50%, was observed for the Ara h2 peptide signal, while suppression of the Ara h3/4 peptide signal was less than 10%. A more recent study by Mattarozzi et al. obtained recoveries between 95 and 118% for lupin β-conglutin peptide in spiked biscuits [77]. Although less expensive than other approaches, this approach requires a calibration curve for each matrix.

Modified synthetic peptide approach: Zhang et al. introduced an internal standard peptide (KILDKVGINNYWLAHKALCSE) with an added asparagine residue (N) in the β-casein peptide VGINYWLAHK. They obtained recoveries between 98.8 and 100.6% [78]. The use of an internal standard allows better recovery, but adding an amino acid can change the retention time and modify the ionization of target peptides.

Standard addition: This label-free quantification strategy consists in adding standards to the matrices. It was used by Posada-Ayala et al. for the quantification of commercial food products [79]. This approach consists in adding different known quantities of extracted allergen proteins directly to the sample to be analysed before digestion and in quantifying the target allergens with the resulting calibration curve. The recovery was not specified, but this approach allows correcting at least for digestion and matrix effects. However, the theoretical level of contamination in the samples must be known in order to adapt the quantities of standards to be added.

3.2. Stable isotope labelling quantification

This strategy is based on the use of isotope-labelled (^{13}C-, ^{15}N-, D-labelled) peptides or proteins [80] (**Table 4b**). It is recommend to use a 6-Da mass difference with respect to the amino acid for doubly charged precursors and an 8–10-Da mass difference for triply charged precursors [52]. Although more expensive than the strategies described above, this approach has the advantage that the unlabelled and isotope-labelled peptides show similar ionization and similar mass spectrometry response signals. For allergen quantification, three kinds of isotope-labelled standards exist [81]: proteins [82], concatemers [83] (or long isotope-labelled peptides) and Aqua peptides [61, 71, 75, 84] (isotope-labelled peptides) (**Figure 5**).

Isotope-labelled proteins: The principle of this approach is to add a labelled protein to the sample before extraction. Newsome et al. studied the recovery of the milk allergen α-S1 casein in baked cookies using a labelled internal α-S1 casein, and obtained recoveries ranging from 60 to 80% [82]. Use of an internal standard allows correcting for the matrix effect and for effects linked to different steps in the sample preparation protocol (protein extraction and enzymatic digestion). It thus allows accurate determination of the recovery and precision for processed samples. This 'gold standard' approach is really expensive, however, making its use unrealistic for the vast majority of routine laboratories.

Isotope-labelled peptides: The principle is to add labelled peptides to the sample after digestion and before the purification steps. This approach is less expensive than the use of isotope-labelled proteins, and synthetic labelled peptides can easily be commercially obtained. Huschek et al. used isotope-labelled peptides to quantify soy, lupin and sesame allergens [59]. They determined the recovery of their method by spiking wheat, cookie and bread with the labelled peptides and obtained results between 69.4 and 112.9%. One could argue, however, that very similar matrices were used in this study (wheat-based products) and that this type of study should be extended to other matrices in order to validate the ability of the isotope-labelled peptide to correct for matrix effects.

Lutter et al. quantified milk proteins in baby food, infant cereals, breakfast cereals and rinsing water, using a calibration curve obtained by spiking 0.1% formic acid with milk protein. The estimated recovery rates were between 16 and 66% [71] Lutter et al. In this study, the isotope-labelled peptides were used to correct for effects related to different steps of the analysis. While using a single calibration curve can be useful in the routine laboratory context, the relatively low recoveries obtained in this study reveal the inability of an isotope-labelled peptide to correct for sample-preparation-related effects. We have compared the areas of milk, egg, peanut and soy peptide peaks for three matrices with and without isotope labelled peptides. Our results clearly show that an isotope-labelled peptide is able to correct for matrix effects but not for effects linked to the extraction and digestion steps [85] planque et al.

Isotope-labelled concatemers/long isotope-labelled peptides: The isotope-labelled concatemer used in this technique is a chimeric protein containing all the labelled target peptides.

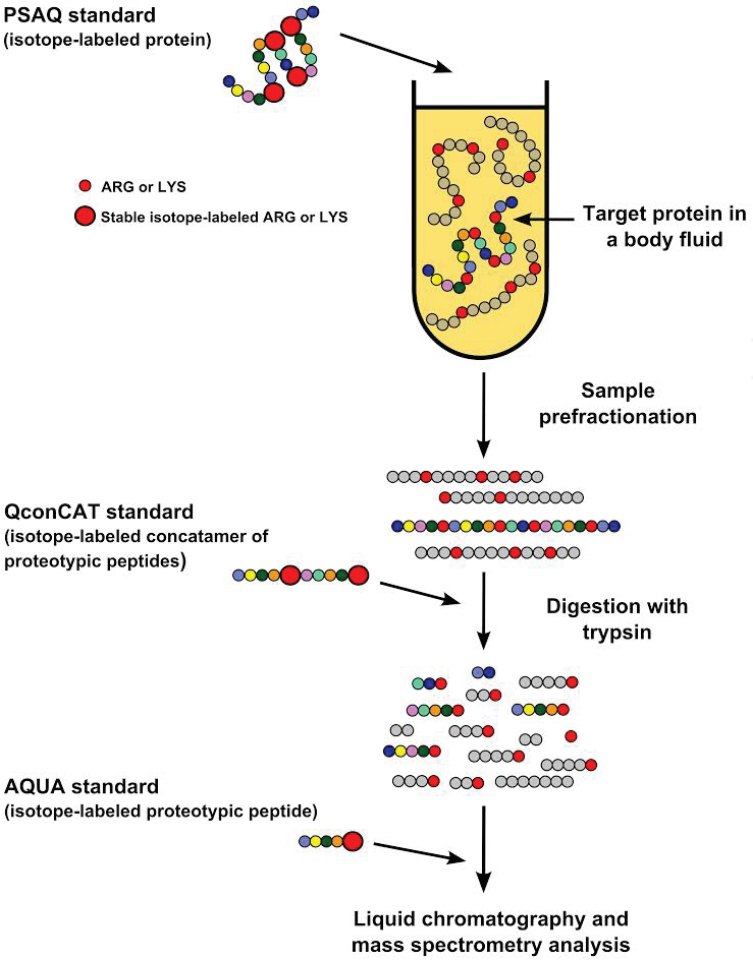

Figure 5. Three types of internal standards are used for the quantification of proteins by mass spectrometry (1) isotope-labelled protein (2) Isotope-labelled concatemers or long isotope-labelled peptides (3) isotope-labelled peptide (from Ref. [81]).

This internal standard is added to the sample before enzymatic digestion. The advantage of this method is that a single concatemer can contain peptides belonging to different proteins or allergens. This strategy has been used in proteomics, but it is not yet used for food allergen quantification [86]. An emerging alternative to use of a concatemer is use of a so-called 'long isotope-labelled peptide'. Chen et al. compared the use of three types of internal standard: human β-casein, isotope-labelled peptide VL [$^{13}C_6$, ^{15}N] PV[$^{13}C_5$, ^{15}N]PQK and a long isotope-labelled peptide QSVLSLSQSKVL[$^{13}C_6$,^{15}N] PV[$^{13}C_5$,^{15}N]PQKAVPYPQRQ [83]. The long isotope-labelled peptide provided better recovery, due to correction for digestion-step-related effects. The recovery based on spiked materials was between 98.8 and 106.7%. In 2016, it was

shown that long isotope-labelled peptides allow recoveries of 97.2–102.5% for α-lactalbumin and 99.5–100.3% for β-casein in the quantification of human milk [87]. This strategy is a good compromise between isotope-labelled proteins and peptides. It allows correcting both for the matrix effect and for digestion-step effects, unlike the use of isotope-labelled peptides.

In conclusion, these studies show that using an isotope-labelled protein or a long isotope-labelled peptide provides better recovery than the isotope-labelled peptide approach. As explained below in the section devoted to result validation, the recovery must be determined with allergen-spiked samples and processed matrices in order to meet AOAC specifications. Published methods, however, do not always meet the AOAC requirements, even with spiked samples. For instance, Careri et al. [76] observed a suppression effect between 30 and 50% for the Ara h2 peptide signal, and Monaci et al. [75] obtained recoveries ranging from 68 to 79% for α-lactalbumin and β lactoglobulin. Altogether, these works show that internal standards are needed for the quantification of allergens in food matrices. Currently, furthermore, the use of a calibration curve for each type of sample is the best way to respect the AOAC guideline requiring a recovery between 60 and 120%.

Future studies should thus still be done to improve the quantification of allergens from a single calibration curve with a good recovery.

4. Validating food allergen methods

While mass spectrometry methods are increasingly sensitive, there remains room for improvement. Furthermore, there subsist obstacles to the harmonization of allergen detection methods in food laboratories [85]. In April 2016, the AOAC SMPR 2016.002 guideline 'Standard method requirements for the detection and quantification of selected food allergens' was published. This guideline is the first to specify target limits for sensitivity and range of linearity, target matrices and reference materials for the analysis of allergens (egg, milk, peanut and hazelnut) in food matrices by mass spectrometry (**Table 5**).

To obtain comparable results among laboratories, it is crucial to adopt validation guidelines. The AOAC guideline, however, is not sufficiently detailed, and each laboratory tends to apply its own rules. In what follows, we compare this guideline with published methods in terms of sensitivity, range of linearity, recovery and precision.

Sensitivity: In the AOAC guideline, the method quantitation limit (MQL) is defined as MQL = average (blank) + 10 x s_0 (blank). Laboratories, however, often use other strategies to determine the limit of quantification (LOQ), such as determining a signal-to-noise (S/N) ratio which should be higher than 10 [56, 60] or estimating an LOD and an LOQ as 3s/slope and 10s/slope, respectively, where s is the standard deviation of the blank signal (n = 10) [57]. On the other hand, the sensitivity can differ from one matrix to another. For example, in a study where cookie, ice cream and sauce were spiked with 0.5 mg milk proteins per kg, the observed S/N ratio was 26 for the cookie matrix, 83 for ice cream and 228 for sauce [85]. This also highlights the importance of a 'fit-for-purpose' description of

an analytical method. Moreover, the sensitivities of developed methods should be determined on the same reference materials (MoniQa, LGC) to ensure (1) their capacity to reach the sensitivity set by the AOAC guideline and (2) an appropriate comparison of method performances.

Linearity: The range of linearity is set as 0.001 to 0.1% allergen contamination (10 mg to 1000 mg) of allergenic ingredients per kg) and thus does not always include the MQL (e. g. an MLQ_{egg} of 5 mg per kg). In the case of high-sensitivity methods, the coefficient of regression is determined using a lower range of concentrations [57, 71].

Recovery: Recovery must range from 60 to 120%. Such recovery values are hard to reach for the detection of allergens in processed samples, and recovery can only be determined by spiking food matrices with allergens. Focusing on egg, milk and peanut in spiked and incurred muffin and cereal bars, Parker et al. constructed calibration curves by spiking the matrices with allergen proteins [61]. In the case of spiked muffin, the determined recovery was 98.6% for egg peptide (GGLEPINFQTAADQAR), 87.7% for milk peptide (YLGYLEQLLR) and 100.2% for peanut peptide (SPDIYNPQAGSLK). When the muffins were baked for 48 min at 177°C, the recoveries were dramatically lower: respectively 45.2%, 75.2% and 70.2%.

Inter- and intra-day coefficients of variation: According to AOAC SPMR, three unknown samples should be analyzed at least seven times to determine the reproducibility of the method. Lamberti et al. determined an intra-day coefficient of variation between 5 and 20% by performing three independent extractions at two different concentrations and three injections per extract [57].

Guidelines for the validation of mass-spectrometry-based methods for allergen analysis should be more precise, like the guidelines 2002/657/EC 'Validation of residues in products of animal origin' and SANCO/12574/2013 'Residues in products of animal origin method validation procedures for pesticide residues analysis in food and feed'. In SMPR 2016.002, several details are missing:

- The number of target peptides that a method should include to confirm the presence of an allergen, as well as fragment ion number and/or type.
- Criteria for the relative retention time, the ion ratio and the specificity of the method (blast, analysis of different blank and matrices), the level of spiking for determining precision and accuracy (for example, the LOQ, action limit and upper limit).

5. Conclusion

The major increase of the allergic population has prompted the development of numerous allergen detection methods. Over the past few years, improvements in the detection of allergens by mass spectrometry have been impressive, allowing detection of processed allergens with high sensitivity (a few mg of proteins per kg of food). Optimization of extraction and purification steps has notably played a key role in the improvement of analytical methods. Allergen quantification is performed mainly with labelled internal standards.

The best approach involves the use of labelled proteins, allowing correction for effects occurring throughout the sample preparation protocol. The high cost of labelled proteins, however, has promoted the use of other strategies, such as methods based on long isotope-labelled peptides and standard addition of allergens.

The validation of qualitative and quantitative MS-based methods for routine detection of allergens is still very recent. The AOAC guideline is a first step towards the development of methods that will allow procedure harmonization, making it possible to compare results between laboratories. These methods should be both improved and extended to other allergens in order to demonstrate their validity and robustness.

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OBJECTIVES OF THE THESIS

The main objective of this work was to develop methods based on ultra-high-performance liquid chromatography coupled to tandem mass spectrometry (UHPLC-MS/MS) for the detection and quantification of peptides from multiple allergens in processed foods. We aimed to develop a method that could be used in a routine laboratory, initially as a tool for confirming the results of ELISAs. We aimed for high sensitivity, in order to protect allergic consumers.

Currently, allergen detection and quantification are widely performed by ELISA. Different food products can yield highly variable results, however, because of matrix interferences. Detecting allergens in processed food products is particularly problematic when an ELISA is used, because thermal processing decreases protein extractability and causes protein denaturation, thus decreasing the detection sensitivity. The allergenicity of a given allergen can be decreased or increased by the thermal process. All this makes it essential to develop reliable analytical methods for detecting allergens in processed foodstuffs. ELISA methods target proteins, while UHPLC-MS/MS methods targets peptides. The selection of marker peptides specific to each allergen in processed and unprocessed food products will ensure the detection of allergens in a large number of food products.

We wanted to develop a single method capable of detecting and quantifying peptides from milk, egg, soy, peanut, almond, pistachio, pecan, cashew, hazelnut and walnut in several food products (cookies, ice cream, chocolate and ice cream), so as to reduce analysis costs for industrialists striving to improve the management of accidental contaminations. Risk assessment methods such as HACCP encourage the use of analytical methods to control contamination. An ELISA can provide a result for only one allergen per analysis, while UHPLC-MS/MS could detect in a single protocol the presence of all 14 allergens whose labeling is mandatory under Regulation 1169/2011/EC.

We have focused in this project on milk, eggs, soy, peanuts, and tree nuts, because they are responsible for the majority of allergic reactions. The food product used to develop the method was cookie, because it is processed at high temperature and because it contains fat (oil) and sugar, and the sugar can generate structural modifications through Maillard reactions. According to VITAL, which fixed thresholds to protect at least 95% of the allergic population, the target limit of quantification (expressed per kg) should be lower than 0.75 mg for egg proteins, 2.5 mg for milk or tree nut proteins, 5 mg for peanut proteins, 25 mg for soybean proteins, and 50 mg for cashew proteins (portion size: 40 g).

CHAPTER I

ADVANCES IN ULTRA-HIGH PERFORMANCE LIQUID CHROMATOGRAPHY COUPLED TO TANDEM MASS SPECTROMETRY FOR SENSITIVE DETECTION OF SEVERAL FOOD ALLERGENS IN COMPLEX AND PROCESSED FOODSTUFFS.

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Context

Food industries increasingly resort to laboratories for the detection of allergens in food products. Recognizing that industrial processes lead to hidden allergen contaminations, they have set improvement goals in order to protect allergic customers and to avoid expensive food recalls.

To achieve these goals, laboratories are urged to develop methods for allergen detection, notably methods based on the enzyme-linked immunosorbent assay (ELISA) and the polymerase chain reaction (PCR). However, ELISAs cannot always detect proteins of allergens in processed food products because the thermal process causes degradation of protein structure (this is especially true of egg allergens). This is why we have developed a UHPLC-MS/MS-based method targeting peptides instead of proteins in order to allow detection of processed allergens that one would otherwise fail to detect because of denaturation of proteins.

The next section concerns the development of a routine method for detecting marker peptides of egg, milk, peanut, and soy allergens by UHPLC-MS/MS in processed food products. This work has been published. Detection of marker peptides for whey proteins and egg yolk allergens, which can easily be separated, respectively, from caseins and egg white, was also included in the method.

The initial target food product in this project was cookie, even though several studies have already targeted this matrix. In some studies the allergens were added after (Weber et al., 2006; Monaci et al., 2014 a) and in others, before the thermal process (Newsome et al., 2013; Chen et al., 2015 a; Gomaa et al., 2015). To the best of our knowledge, only two sensitive methods for multi-allergen detection in processed food products were published before 2015. The limits of detection (LODs) (S/N>3) reached with the method developed by Heick et al. (2011) for milk, egg, soy, and peanut were respectively 5, 42, 24, and 11 mg of soluble proteins per kg of bread (200 °C-60 min) (Heick et al., 2011). Gomaa et al. (2015) obtained a limit of quantification (LOQ) (S/N>10) of 10 mg casein or soy proteins per kg cookie matrix (cooked at 177 °C for 12 min) (Gomaa et al., 2015). In other words, the LOQs reached with these two methods in processed cookies or bread are in most cases well above the corresponding VITAL thresholds (expressed in mg of total proteins per kg), set at 2.5 mg milk proteins, 0.75 mg egg proteins, 25 mg soybean proteins, and 5 mg peanut proteins per kg (portion size: 40 g).

The comparison between method performances is rather difficult due to the lack of allergen reference materials and the differences in the units to express results (mg of soluble proteins, mg of ingredients). In this study, we expressed results in mg of total proteins per kg of foods, based on the

theoretical content of proteins in raw ingredients as it was determined by VITAL to fix the different thresholds for each allergen.

To increase the originality and value of this work, three food matrices were added: tomato sauce (acidic and slightly processed), chocolate (tannins), and banana ice cream (fat). The detection of allergens in two processed matrices (cookie and sauce) and two complex ones (chocolate and ice cream) established the suitability of the method for detecting allergens in a wide range of food products.

Major achievements

The detection of marker peptides of four major allergens (milk, egg, soy and peanut) in four complex or processed matrices (cookie, tomato sauce, ice cream and chocolate) by UHPLC-MS/MS with a high sensitivity makes a significant improvement to the state of art. After the selection of abundant marker peptides in raw and processed ingredients by UHPLC-MS/MS, the method was optimized using processed cookies (180°C-18 min) containing 1350 mg of milk proteins, 3250 mg of egg proteins, 2100 mg of soy proteins, and 1250 mg of peanut proteins per kg of cookies (expressed in mg of total proteins per kg). Afterwards, incurred matrices were prepared at several concentrations of allergic foods in order to experimentally determine a single LOQ defined by a signal to noise ratio higher than 10 and 3 for the first and the second transitions, respectively, for the four target matrices. The sensitivities reached in this study (LOQ) are 3.4 mg and 30.8 mg egg proteins (white and yolk proteins, respectively), 0.5 mg and 5 mg milk proteins (casein and whey proteins, respectively), 2.5 mg peanut proteins, and 5 mg soybean proteins per kg.

The LOQs for milk (casein), soy, and peanut reached by our method were below the VITAL thresholds, but the LOQ for egg was still 4.5 times as high. Yet this threshold of 3.4 mg total egg proteins per kg cookie (LOQ) was much lower than the 42 mg soluble egg proteins per kg (LOD) reached by Heick et al. (2012) in processed bread. It thus represents a real step forward.

Preliminary optimization of the method

The selection of marker peptides is the first step for the development of UHPLC-MS/MS methods. The amino acid sequence of milk, egg, soy or peanut proteins are well characterized in databases, hence an *in silico* approach was used to identify target marker peptides.

Fist, the target proteins were selected from Uniprot (http://www.uniprot.org/) and an *in silico* digestion was performed using the open source software Skyline (https://skyline.ms/). The *in silico* digestion of proteins generated a list of peptides and multiple reaction monitoring (MRM) transitions depending on criteria set by the user such as the digestive enzyme (trypsin), the peptide length (8 to 25 amino acids), the charge state of peptides (2+, 3+) and transitions (1+, 2+), modifications (missed cleavages, carbamidomethylation of cysteines), or the fragmentation (b, y). Several hundred peptides per allergen were generated after *in silico* digestion of milk, egg, soy and peanut proteins. A first selection was done by analyzing peptides after a tryptic digestion of protein extracts of raw ingredients (0.1 mg of proteins per ml of 200 mM of TRIS-HCl; pH 8.2). Only peptides with most MRM signals at the same retention time (RT) were conserved and 3 to 4 abundant MRM transitions were kept per peptide. This first step had considerably decreased the number of peptides and MRM transitions. However, the goal of this method is to detect peptides of allergenic foods by UHPLC-MS/MS in highly processed food products. Consequently, incurred cookies containing 1350 mg of milk proteins, 3250 mg of egg proteins, 2100 mg of soy proteins, and 1250 mg of peanut proteins per kg of cookies were processed at 180°C during 18 min. After the extraction of proteins from cookies in a buffer (composition: 200 mM TRIS-HCl; pH 8.2, 5 M urea) the digestion of proteins with trypsin, samples were analyzed in order to select peptides that were robust to the thermal process. A basic local alignment search tool (BLAST) was performed to ensure the specificity of the amino-acid sequence of the target peptides.

To reach VITAL thresholds, it was essential to optimize several parameters, such as the extraction and purification conditions. After the optimization of the method described in the following paragraphs, milk, egg, soy and peanut ingredients were incurred in four target complex and processed matrices (cookie, tomato sauce, ice cream and chocolate). The most abundant and specific peptides (BLAST and analysis of allergen-free matrices) of milk, egg, soy and peanut proteins in the four target matrices were conserved and published.

I Optimization of the extraction method

The goal of the optimization of extraction method was to obtain the best extraction yield of proteins, based on the theoretical content of proteins in homemade cookies, and the highest peak area for target marker peptides after an analysis of cookies by UHPLC-MS/MS, keeping in mind the required ease of use for routine laboratories. This optimization was the first step toward the development of a sensitive detection method that will enable to achieve the sensitivity fixed by VITAL.

The optimization was done at the beginning of the project, while the final list of target peptides was not yet established. Consequently, the main difficulty of this optimization was the high number of MRM data obtained by UHPLC-MS/MS after the analysis of more than 100 peptides for the four target allergens. Moreover, the physicochemical properties of proteins/peptides such as their hydrophobicity might influence the extraction and the digestion of proteins and the analysis of peptides. Hence, proteins/peptides could be differently influenced by the tested parameters triggering the search of compromises to obtain the best extraction yield of proteins and the highest peptide peak area for the majority of target peptides.

In order to simplify and highlight the influence of the different parameters tested on the peak area of the target marker peptides of milk, egg, peanut and soy proteins, only the twenty peptides of the final method will be presented.

The extraction parameters were chosen by analyzing three independent preparations of incurred cookies (180 °C – 18 min) containing 1350 mg of milk proteins, 3250 mg of egg proteins, 2100 mg of soy proteins, and 1250 mg of peanut proteins per kg of cookies. The theoretical protein content of 2 g cookie was 125.4 mg proteins, including 109.5 mg wheat proteins, 2.7 mg milk proteins, 6.5 mg egg proteins, 2.5 mg peanut proteins, and 4.2 mg soy proteins.

The optimization of the different extraction parameters was done step by step according to the information retrieved from the literature and from the results progressively obtained during the optimization of the method.

Parameters tested in order to optimize protein extraction from cookies:

- **Extraction buffer:** (1) 200 mM Tris-HCl, pH 8.2; (2) 50 mM Tris, 500 mM NaCl, pH 8.2, (3) 50 mM PBS, pH 7.4, and (4) 50 mM NH_4HCO_3, pH 8.2.
- **Extraction buffer pH:** 7.2, 8.2, and 9.2.
- **Extraction temperature:** 20 °C and 60 °C.

- **Tested detergents and chaotropic agent:** Triton X-100, SDS (sodium dodecyl sulfate), CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate), and urea.
- **Urea concentration:** 0, 1, 2, 3, 4, and 5 M.
- **Extraction time:** 0.5, 1, 2, and 3 h.
- **Ratio of sample weight to extraction buffer volume:** extraction of proteins from 2 g cookies with 5, 10, 20, or 40 mL extraction buffer.
- **Defatting:** flushing with acetone prior to extraction.

Evaluating the best extraction parameters

Optimal extraction parameters were selected on the basis of two criteria.

First, the BCA (BiCinchoninic acid Assay - ref: 23225 - Thermo Fisher Scientific) was used to measure the protein content after extraction. This assay combines the reduction of copper ions (Cu^{2+} to Cu^{+}) by proteins with the detection of Cu^{+} with bicinchoninic acid. Cookie protein extracts were diluted 2 to 8 times in the tested extraction buffer and incubated with BCA reagents A and B for 30 min at 37 °C to determine the protein concentration by measuring the absorption in a spectrophotometer and using a calibration curve. Calibration curves spanning the concentration range 0 to 1 mg Bovine Serum Albumin (BSA) proteins per mL were prepared in extraction buffer and the absorbance was measured at 562 nm.

Secondly, the protein extracts diluted to 1 mg/mL were digested and analyzed by mass spectrometry (Acquity UHPLC – Xevo TQS triple quadrupole - Waters) in order to assess, by comparing peptide peak areas, the effect of extraction buffer composition on the detection of peptides related to the target allergens of interest. A logarithmic scale was applied because of the great peak area differences between peptides.

I.I Selection of the extraction buffer

Four extraction buffers, which are often used for food protein extraction in the literature (Heick et al., 2011; Gomaa et al., 2015)were tested: (1) 200 mM Tris-HCl, pH 8.2; (2) 50 mM Tris, 500 mM NaCl, pH 8.2, (3) 50 mM PBS, pH 7.4, and (4) 50 mM NH_4HCO_3, pH 8.2 Extraction buffer (10 mL) was used to extract the proteins contained in 2 g incurred cookies (n=3). Extraction was performed for 1 h and was followed by centrifugation at 4660 × g for 20 min at 20 °C.

The extraction yield was determined for each of the buffers described above. A calibration curve spanning the concentration range 0 to 1 mg BSA proteins per mL was prepared in each extraction buffer. The corresponding experimental extraction yields are presented in **Figure 23**.

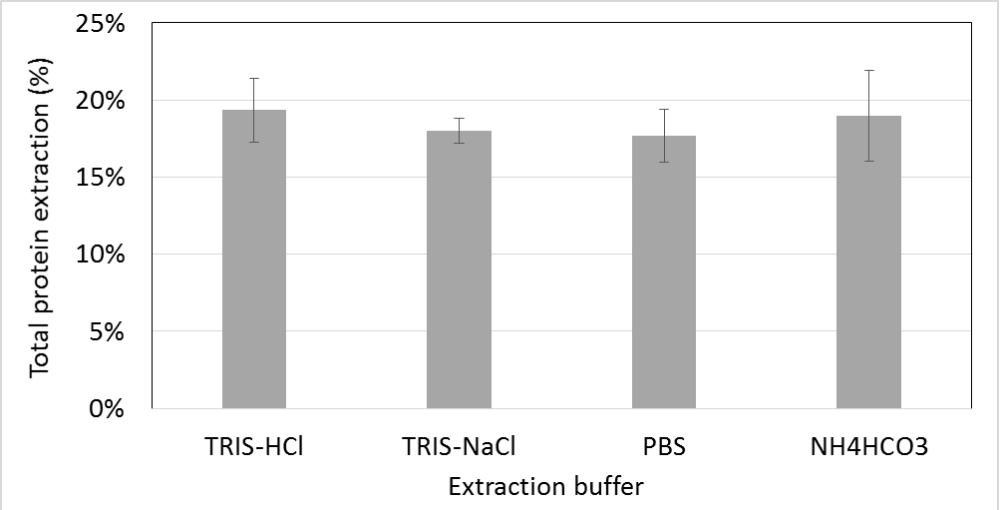

Figure 23: *Effect of the extraction buffer on the extraction yield of proteins contained in 2 g cookie. The extraction buffers used were: (1) 200 mM Tris-HCl, pH 8.2; (2) 50 mM Tris, 500 mM NaCl, pH 8.2, (3) 50 mM PBS, pH 7.4; and (4) 50 mM NH_4HCO_3, pH 8.2. Results are expressed as mean protein extraction yields (%) (± 1 S.D. (n=3)). Statistical analysis was performed with Student's t-test and no significant difference in protein extraction yield was observed between extraction buffers. An extraction yield of 100% corresponds to 12.54 mg proteins/mL (2 g cookie in 10 mL buffer).*

No significant difference in protein extraction yield was observed between buffers. The protein extraction yield is weak. Indeed less than 20 % of theoretical proteins in cookies were extracted. This weak extraction yield can be, most likely, explained by the difficulty of protein extraction due to aggregation during the thermal process and the presence of insoluble proteins.

To compare the marker peptide peak areas obtained by UHPLC-MS/MS, each extract was diluted in 200 mM NH_4HCO_3 to 1 mg proteins/mL. One hundred microliters of sample were added to 100 µL of 200 mM NH_4HCO_3. Protein reduction was performed by adding 100 µL of 200 mM DTT for 45 min, followed by alkylation with 80 µL of 500 mM IAA for 45 min in the dark. Samples were diluted with 100 µL of 50 mM NH_4HCO_3 before the digestion with 20 µL trypsin (Promega – modified trypsin T5111) (0.1 mg/mL). The reaction was stopped with 30 µL of 2% formic acid. The three biological samples extracted with each of the four extraction buffers were analyzed by UHPLC-MS/MS. The mean peptide peak areas obtained for each extraction buffer are presented in **Figure 24**.

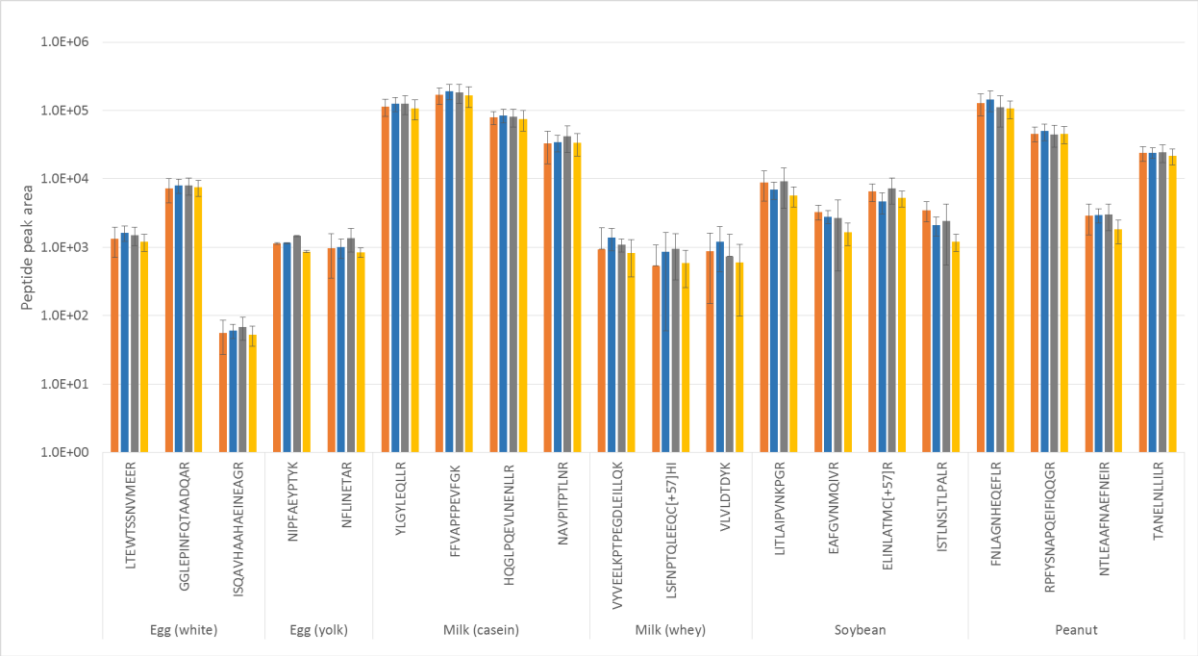

***Figure 24**: Extraction buffer effects on peak areas (represented on a logarithmic scale). The buffers tested were: (1) 200 mM Tris-HCl, pH 8.2 (orange); (2) 50 mM Tris, 500 mM NaCl, pH 8.2 (blue); (3) 50 mM PBS, pH 7.4 (gray) and (4) 50 mM NH_4HCO_3, pH 8.2 (yellow). Results are expressed as mean areas ± 1 S.D. (n=3) and presented on a logarithmic scale. Statistical analysis was performed with Student's t-test and no significant effect of the choice of extraction buffer on peptide peak area was observed.*

Student's t-test analyses revealed no significant effect of extraction buffer composition on the peak areas corresponding to milk, egg, soy, or peanut peptides. For target marker peptides, we could thus retain any one of the four buffers, and chose to keep 200 mM Tris-HCl, pH 8.2 for protein extraction from cookies. Indeed, the selection of the extraction buffer was based on a higher number of peptides and an improvement of peak areas was previously observed with this buffer.

I.II Optimization of the extraction buffer pH

The pH range for efficient digestion by trypsin is 6.0 to 10.0, and efficiency is described to be higher in the pH range 7.0 to 9.0 (Jung et al., 2014). Three different pH values were tested: 7.2, 8.2, and 9.2.

The protein content was measured with BCA kit and the pH of the extraction buffer did not have any effect on the protein extraction yield (**Figure 25**). The protein extracts were then diluted to 1 mg/mL in 200 mM NH_4HCO_3, digested, and peptides were analyzed by mass spectrometry. No statistically significant difference was observed according to the pH (**Figure 26**).

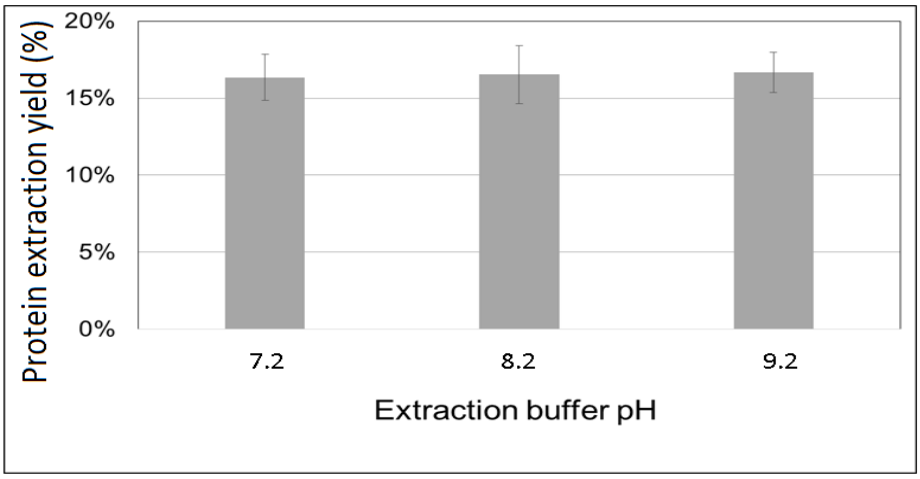

***Figure 25**: Effect of the pH of the extraction buffer (200 mM Tris-HCl) on the extraction yield of proteins recovered from cookies. Results are expressed as mean protein extraction yields (%) ± 1 S.D. (n=3). Statistical analysis was performed with Student's t-test and no significant difference on protein extraction yield was observed according to the pH. An extraction yield of 100% corresponds to 12.54 mg proteins/mL (2 g of cookie in 10 mL buffer).*

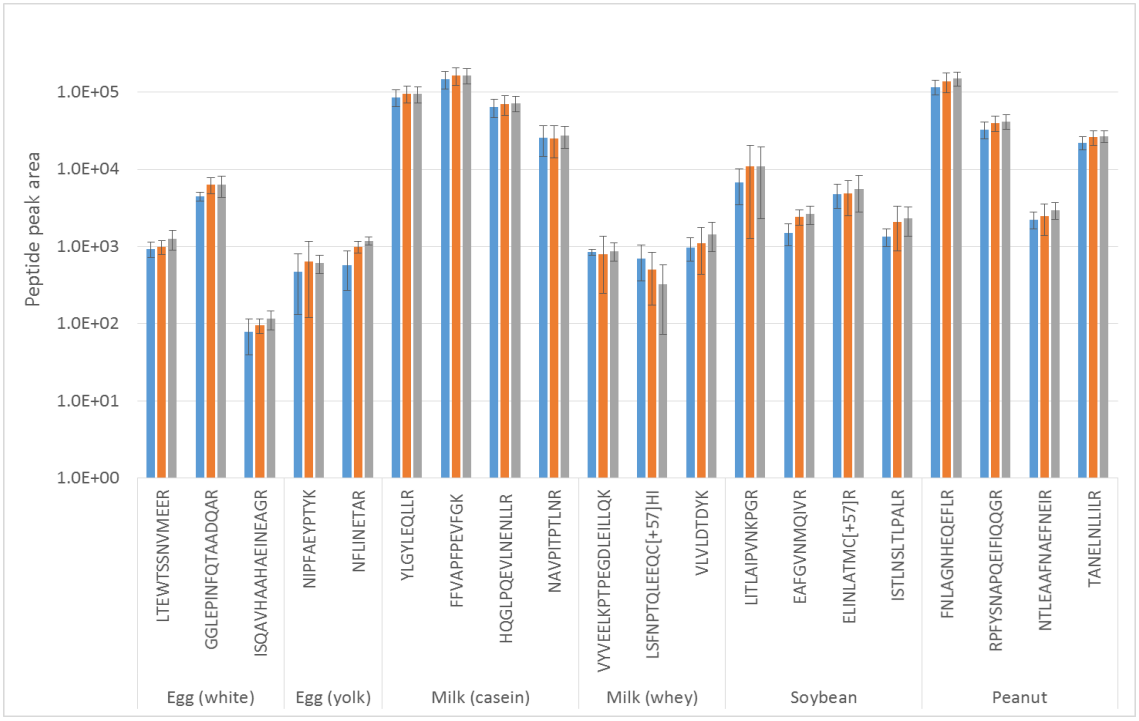

***Figure 26**: Effect of the extraction buffer pH on the peak areas of milk, egg, soy, and peanut peptides extracted with 200 mM Tris-HCl buffer. The different pH tested were 7.2 (blue), 8.2 (orange) and 9.2 (gray). Results are expressed as mean areas ± 1 S.D. (n=3) and represented on a logarithmic scale. Statistical analysis was performed with Student's t-test and no significant differences in peak areas were observed according to the pH.*

As the pH appeared to have no significant effect on the peptide peak areas, any one of the three extaction buffers could be kept. The pH chosen for future experiments was pH 9.2 even though, no improvement was observed. This pH was chosen based on the improvement of peptide peak areas for the analysis of more than 100 peptides as observed for the selection of the extraction buffer.

I.III Optimization of the extraction temperature

It is known that higher temperatures during extraction might improve the protein extraction yield, a phenomenon most likely due to due to the increase in molecule agitation that could promote the solubilization of proteins (Bucić-Kojić et al., 2009; Albillos et al., 2011). Two extraction temperatures were thus tested: 60 and 20 °C with the buffer composed of 200 mM Tris-HCl, pH 9.2.

Extraction at 60 °C was found to have a better yield than extraction at 20 °C (20% vs. 16%) (**Figure 27**).

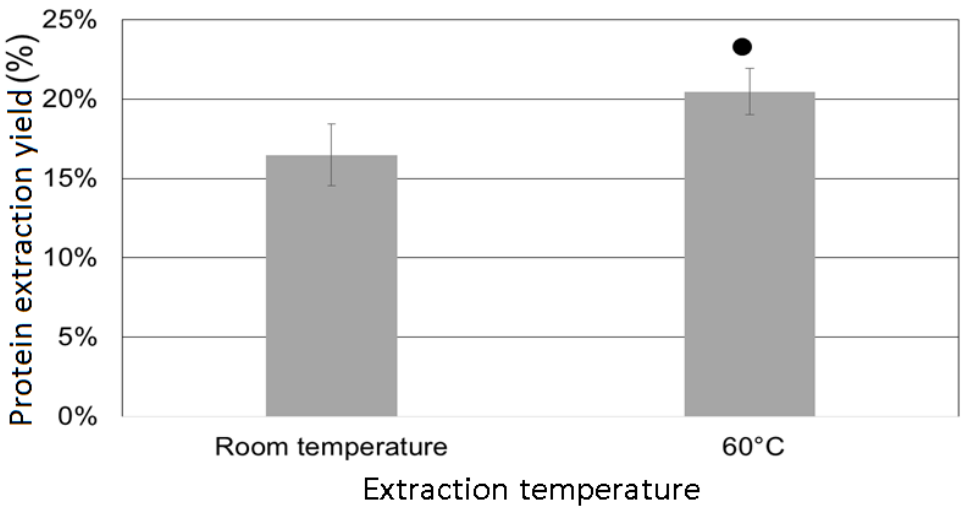

Figure 27: *Effect of the extraction temperature on the protein extraction yield. Proteins were extracted from cookies with 200 mM Tris-HCl, pH 9.2. Results are expressed as mean protein extraction yields (%) ± 1 S.D. (n=3). Statistical analysis was performed with Student's t-test p value: < 0.1 (●). An extraction yield of 100 % corresponds to 12.54 mg proteins/mL (2 g cookie in 10 mL of buffer).*

The protein extracts were diluted to 1 mg/mL in 200 mM NH_4HCO_3, digested, and analyzed by UHPLC-MS/MS. As shown in **Figure 28**, the peak areas corresponding to 4 soy peptides were significantly increased when extraction was carried out at 60 °C.

The extraction yield and peak area increases observed at 60 °C as compared to 20 °C were significant, but for testing the effect of the chaotropic agent urea, extraction at 20 °C (in 200 mM

Tris-HCl pH 9.2) was retained, as heating with urea induces protein modifications (carbamylation by binding of isocyanic acid (HNCO) to N-terminal amino acids and to lysine and arginine residues) (Kollipara et al., 2013; Gillery et al., 2015). The added value of extracting at 60 °C was compared with that of extracting at 20 °C but adding urea to the extraction buffer (see next section - the effect of adding various detergents was also tested at 20 °C).

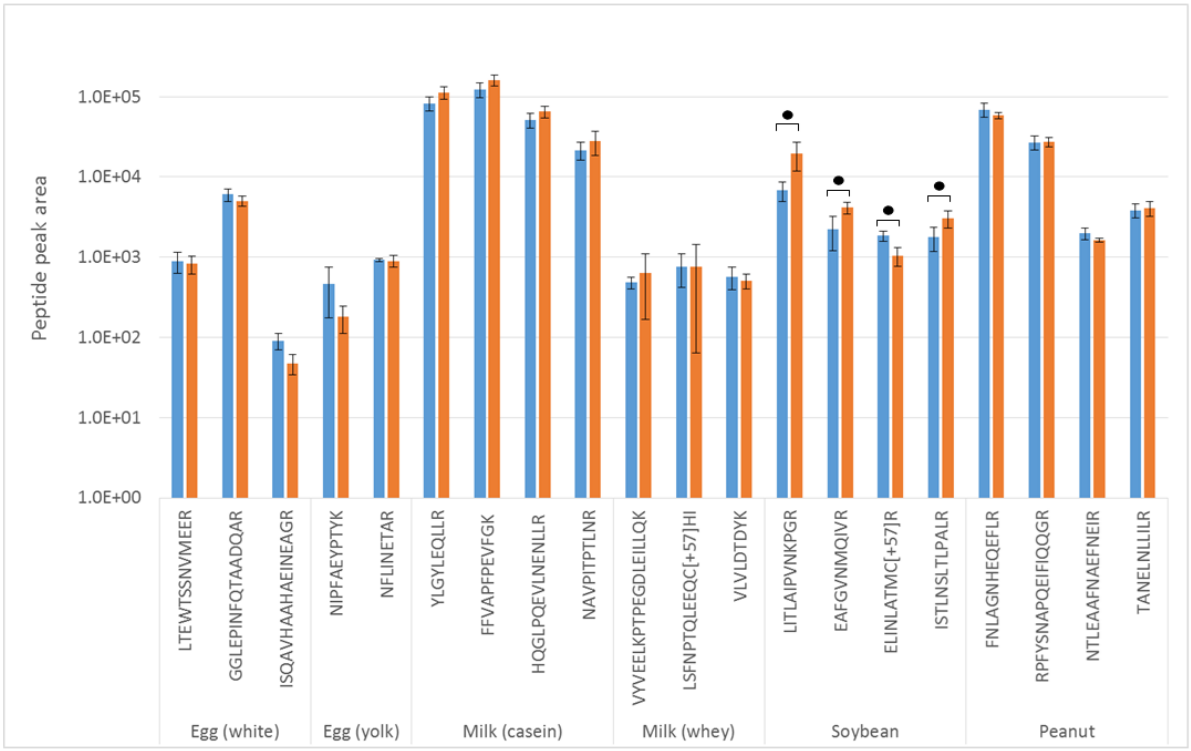

Figure 28: Effect of the extraction temperature (20 °C (blue) or 60 °C (orange)) on the *peak areas of milk, egg, soy, and peanut peptides extracted for 30 min with 200 mM Tris-HCl pH 9.2. Results are expressed as mean areas ± 1 S.D. (n=3) and represented on a logarithmic scale. Statistical analysis was performed with Student's t-test. p value: < 0.1 (●).*

I.IV Use of a chaotropic agent and/or detergents

I.IV.1 Effect of chaotropic agent and/or detergents on protein extraction yield

Additional measures that might be taken to improve protein extraction and digestion include adding a detergent or chaotropic agent. Not all detergents, however, are compatible with mass spectrometry, as they may contaminate MS instruments and interfere with chromatographic resolution, and it is hard to get rid of them (Proc et al., 2010).

One agent we chose to test was the urea, a chaotropic molecule known to disrupt hydrogen bonds between water molecules and to reduce the stability of native proteins by weakening the hydrophobic effect and induce protein denaturation (Salvi et al., 2005). We also selected the detergents Sodium Dodecyl Sulfate (SDS – an anionic detergent), Triton X-100 (a non-ionic aqueous detergent), and CHAPS (a zwitterionic detergent), although these agents must be removed prior to MS analysis (Yeung et al., 2008).

The extraction conditions tested were as follows: extraction buffer alone (200 mM Tris-HCl pH 9.2); , extraction buffer with 5 M urea; extraction buffer with 5 M urea plus either SDS, Triton X-100, or CHAPS at theirs critical micelle concentration (respectively 8.8 mg/mL, 10 µL/mL, and 40 mg/mL). (**Figure 29**).

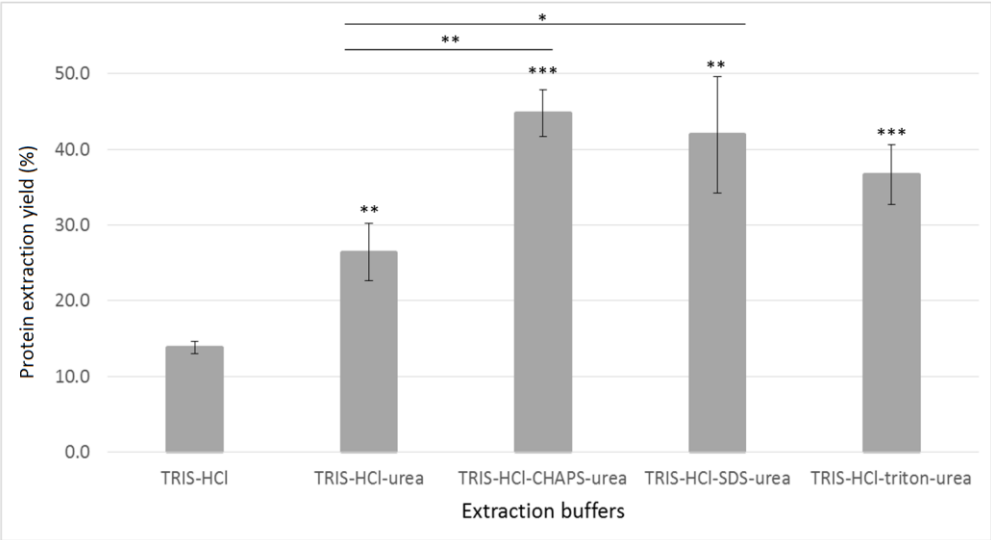

Figure 29*: Effect of adding urea, alone or with a detergent, on the extraction of proteins at 20 °C from cookies. The extraction buffer used was: (1) 200 mM Tris-HCl, pH 9.2; (2) 200 mM Tris-HCl, pH 9.2, 5 M urea, (3) 200 mM Tris-HCl, pH 9.2, 5 M urea plus (4) 40 mg/mL CHAPS (5) 8.8 mg/mL SDS, or (6) 10 µL/mL Triton X-100. Results are expressed as mean protein extraction yields (%) ± 1 S.D. (n=3). Statistical analysis was performed with Student's t-test: p value: < 0.001 (***) < 0.01 (**) < 0.05 (*). An extraction yield of 100% corresponds to 12.54 mg proteins/mL (2 g cookie in 10 mL buffer).*

Adding urea did significantly increase the protein extraction yield, and adding CHAPS or SDS allowed a further significant increase.

I.IV.2 Effect of detergent removal spin column on peptide peak areas

As the tested detergents are not compatible with mass spectrometry and have to be removed, a commercial kit (Pierce detergent removal spin column, 0.5 mL ref: 87777 – Thermo Scientific) was

tested on samples prepared from digested cookies (n=3), extracted with 200 mM Tris-HCl; pH 9.2, 5 M urea. The protein extracts were diluted to 1 mg/mL and analyzed by UHPLC-MS/MS. The spin columns were used just before injection. The extracts were analyzed before and after their elution through the spin column in order to compare peptide peak areas with or without the use of detergent removal spin columns (**Figure 30**). The lack of detergents in this experiment allows the comparison of peptide peak areas.

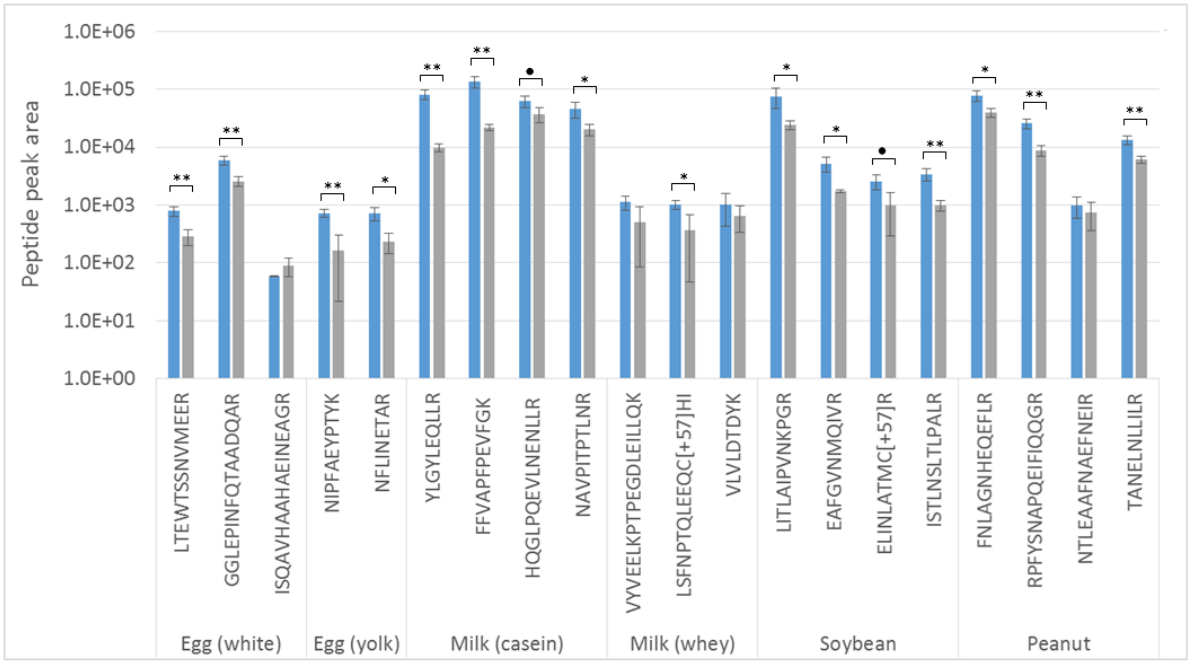

Figure 30: *Effects of clean-up on the peak areas corresponding to milk, egg, soy, and peanut peptides obtained after extraction with 200 mM TRIS-HCl, pH 9.2 containing 5 M urea. After digestion, the extracts were split and injected before (no clean-up, in blue) or after elution through the spin column (Pierce clean-up in gray). Results are expressed as mean areas ± 1 S.D. (n=3) and presented on a logarithmic scale. Statistical analysis was performed with Student's t-test p value: < 0.01 (**) < 0.05 (*) < 0.1 (●).*

One can see that while detergents can improve the extraction yield, the passage through the spin column decreases the peak areas corresponding to 16 of the 20 peptides detected. In conclusion, the use of detergents does not sufficiently increase the protein extraction yield to compensate for the peak area decreases observed after passage through the Pierce detergent removal spin column.

We thus chose not to add any detergent, but we did add urea, as urea treatment at 20 °C proved superior to extraction at 60 °C (**Fig 27** vs. **Fig. 29**), and as adding 5 M urea to the extraction buffer is compatible with mass spectrometry. The impact of urea on the protein extraction yield and peptide peak areas was also optimized.

I.V Optimization of the extraction time

The effect of the extraction time on the protein extraction yield was tested. Extraction was carried out for 30 min, 1 h, 2 h, and 3 h in 200 mM Tris-HCl pH 9.2 containing 5 M urea, and the protein content was determined by BCA. This parameter was evaluated with the goal of improving protein extraction yield and/or shortening the extraction time.

No significant effect of the extraction time on protein recovery was observed (**Figure 31**).

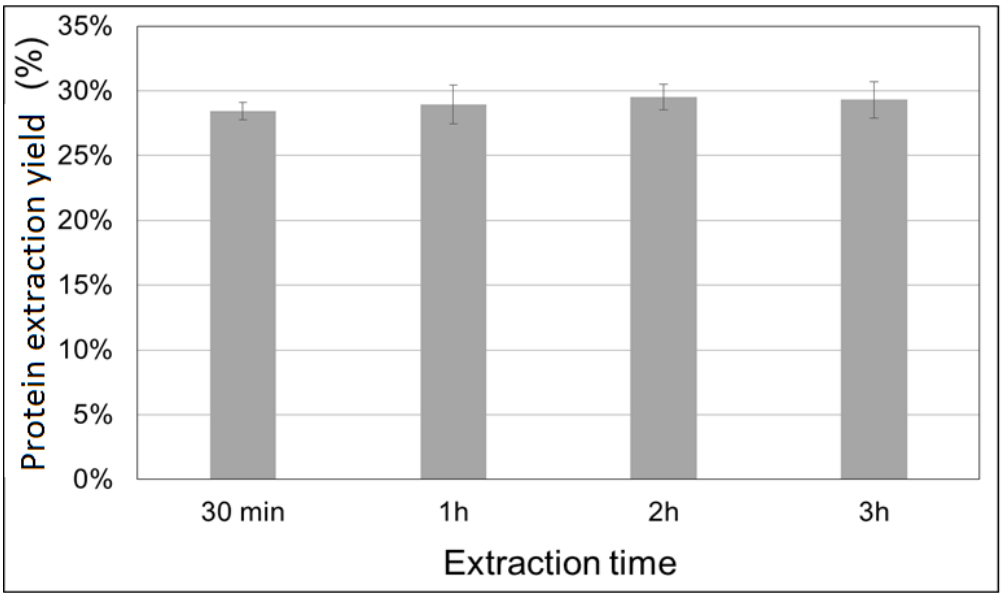

Figure 31: *Effect of different extraction times on the recovery of proteins from cookies. Extraction was carried out for the indicated time in 200 mM Tris-HCl, pH 9.2 containing 5 M urea. Results are expressed as mean protein extraction yields (%) ± 1 S.D. (n=3). Statistical analysis was performed with Student's t-test. No significant difference in protein extraction yield was observed according to the extraction time. An extraction yield of 100% corresponds to 12.54 mg proteins/mL (2 g cookie in 10 mL buffer).*

The protein extracts were diluted to 1 mg/mL in 200 mM NH_4HCO_3, digested and peptides were detected by mass spectrometry (**Figure 32**).

UHPLC-MS/MS confirmed the lack of any significant effect of the extraction time on peptide recovery (**Figure 32**). On the basis of these observations, an extraction time of 30 min was retained in order to develop a routine method as short as possible.

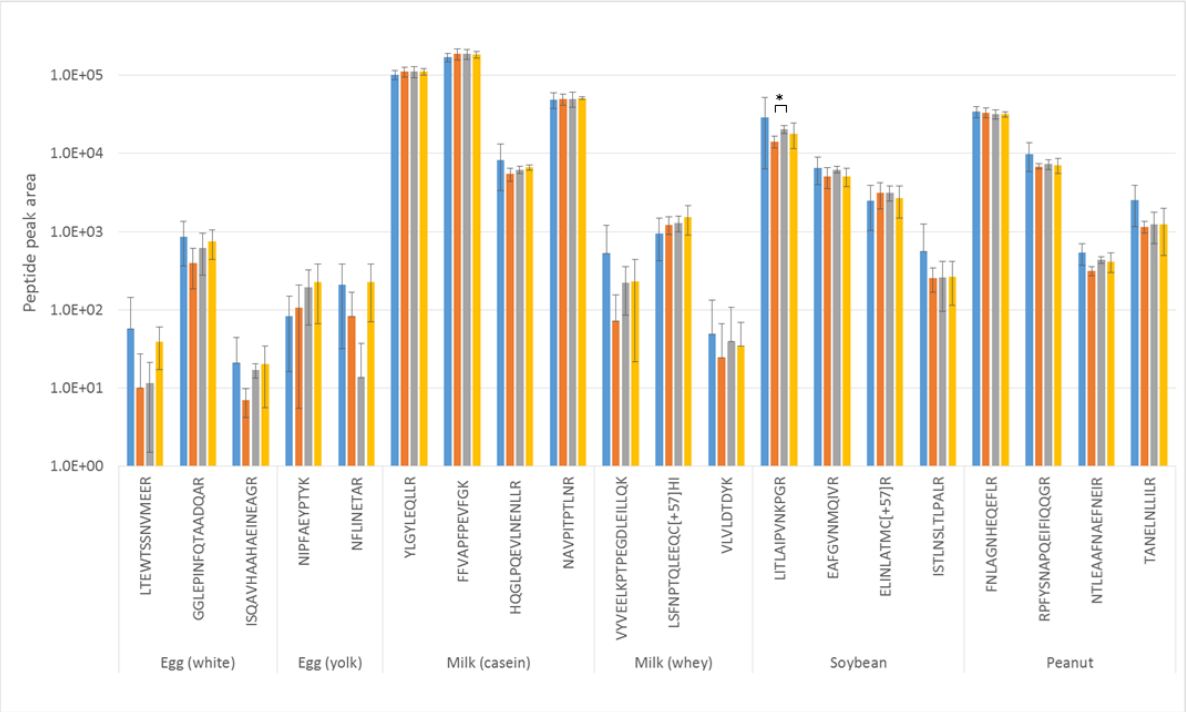

***Figure 32**: Effects of different extraction times on the peak areas corresponding to milk, egg, soy, and peanut peptides. Extraction was performed in 200 mM Tris-HCl; pH 9.2 containing 5 M urea. The extraction time was 0.5 (blue), 1 (orange), 2 (gray), or 3 (yellow) hours. Results are expressed as mean areas ± 1 S.D. (n=3) and presented on a logarithmic scale. Statistical analysis was performed with Student's t-test. p value: < 0.05 (*).*

I.VI Optimization the ratio of sample weight to extraction buffer volume

The ratio of sample weight to extraction buffer volume can have a major influence on protein extraction and can affect extract quality (larger volumes give rise to cleaner extracts). Several sample weight/extraction buffer volume ratios were tested for their effect on the extraction yield (**Figure 33**) and peptide peak areas (**Figure 34**). The quantity of sample material was arbitrarily set at 2 g and extraction was done with 5, 10, 20, or 40 mL extraction buffer (200 mM Tris-HCl; pH 9.2 with 5 M urea).

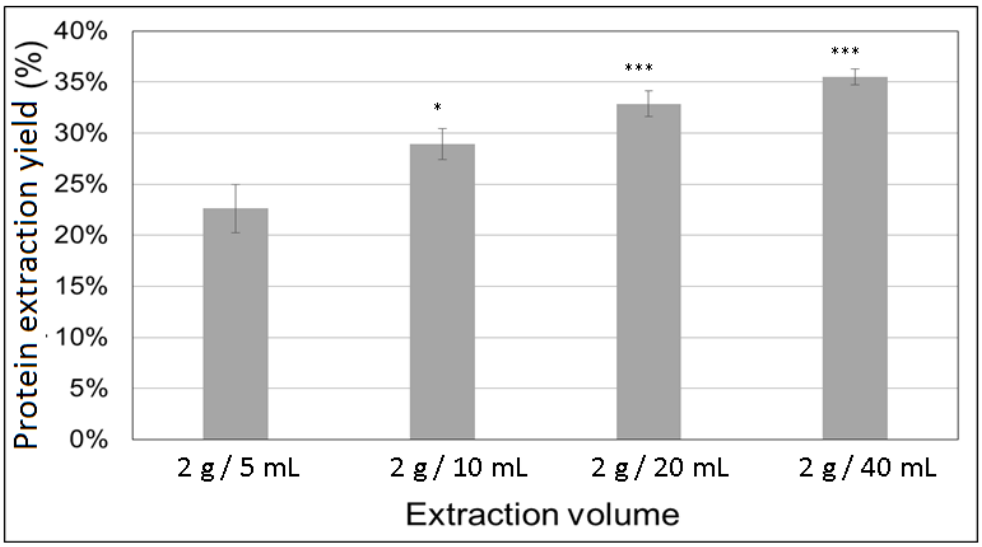

Figure 33: *Effect of the ratio of sample weight to extraction buffer volume on the extraction of proteins from 2 g cookie with 5, 10, 20, or 40 mL extraction buffer (200 mM Tris-HCl; pH 9.2, 5 M urea). Results are expressed as mean protein extraction yields (%) ± 1 S.D. (n=3). Statistical analysis was performed with Student's t-test: p value: < 0.001 (***) < 0.05 (*). For 2 g cookie in 5, 10, 20, and 40 mL, respectively, an extraction yield of 100% corresponds to 25.08, 12.54, 6.27, and 3.14 mg proteins per mL.*

The protein extraction yield was found to increase as the extraction buffer volume increased (**Figure 33**). The concentration of proteins in the extract decreased, however, since the protein concentration decreased as the extract volume increased (from 25.08 mg/mL for 2 g/5 mL to only 3.14 mg/mL for 2 g/40 mL).

The protein extracts were diluted to 1 mg/mL in 200 mM NH_4HCO_3 and analyzed by mass spectrometry.

The UHPLC-MS/MS analysis showed an increased peak area for two egg peptides (GGLEPINFQTAADQAR and NFLINETAR), one milk peptide (HQGLPQEVLNENLLR), two soy peptides (LITAIPVNKPGR and ISTLNSLTLPARL), and two peanut peptides (RPFYSNAPQEIFIQQGR and TANELNLLILR) in response to an increased extraction buffer volume. Yet a decrease was also observed for two milk peptides (FFVAPFPEVFGK and HQGLPQEVLNENLLR) under these conditions (**Figure 34**).

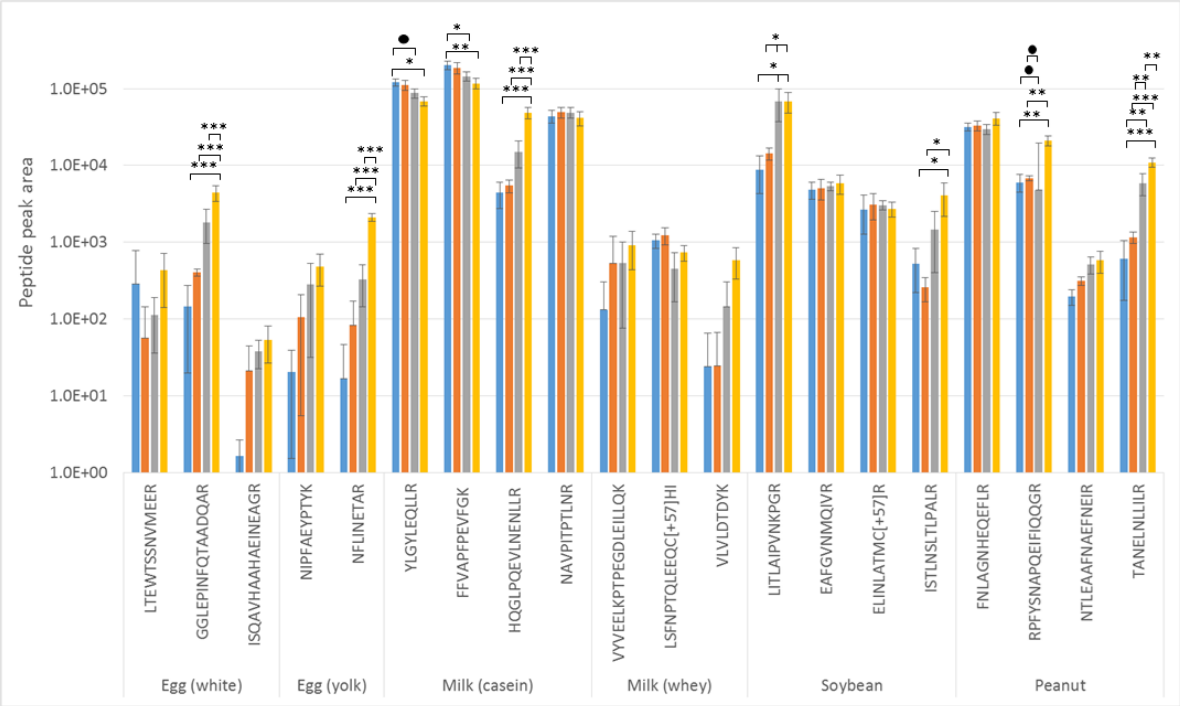

Figure 34: *Effect of the ratio of the sample amount to the extraction buffer volume on the areas of milk, egg, soy, and peanut peptide peaks. Extraction was performed for 30 min with 200 mM Tris-HCl pH 9.2 containing 5 M urea. The amount of sample was set at 2 g and the extraction buffer volume was 5 mL (light blue), 10 mL (orange), 20 mL (gray), or 40 mL (yellow). Results are expressed as mean areas ± 1 S.D. (n=3) and presented on a logarithmic scale. Statistical analysis was performed with Student's t-test p value: < 0.001 (***) < 0.01 (**) < 0.05 (*) < 0.1 (●).*

In conclusion, extract cleanliness was really improved when the ratio was increased to 2 g/20 mL or 2 g/40 mL. In addition, larger peak areas were obtained for 7 of the 20 peptides. To ensure extract cleanliness while maintaining a suitable protein concentration, the ratio chosen for further analyses was 2 g/20 mL. Please note that the concentration of proteins in the extract had an influence because it was eventually decided not to dilute all extracts to 1 mg/mL prior to digestion but to dilute them only 1:1 (v/v) (see next paragraph).

I.VII Optimization of the urea concentration

Several studies have shown a decrease in trypsin activity at urea concentrations above 1 M (Viswanathat et al., 1955; Carpenter, 1967). The effect of increasing the urea concentration in the buffer (200 mM Tris-HCl pH 9.2) from 0 to 5 M was therefore tested for its effects on both protein extraction and peptide abundances (**Figures 35 and 36**). The optimization of the urea concentration was performed in parallel with the optimization of the ratio of sample weight to extraction buffer volume, consequently, a volume of 10 mL of extraction buffer was used.

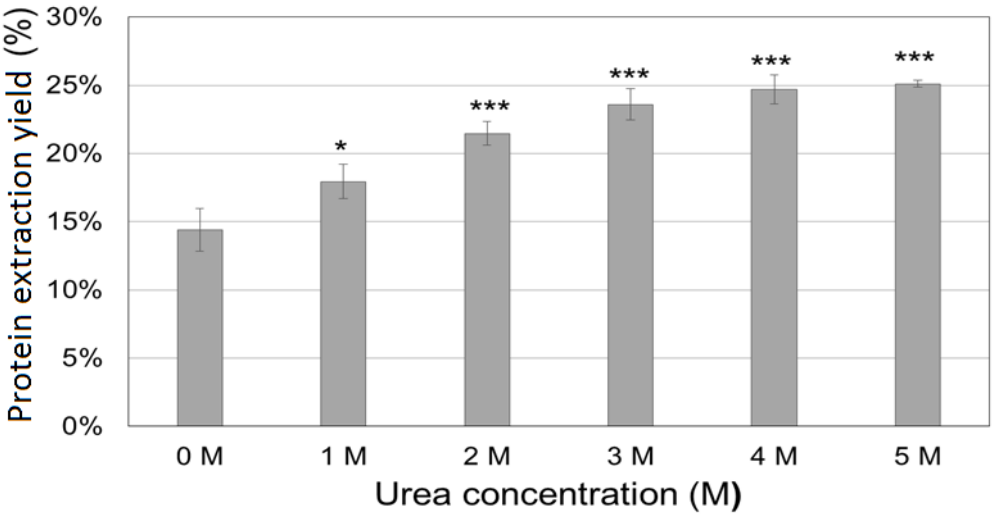

Figure 35: *Effect of urea on the extraction yield of proteins contained in cookies. Extraction was performed for 30 min in 200 mM Tris-HCl pH 9.2 containing urea at 0, 1, 2, 3, 4, or 5 M concentration. Results are expressed as mean protein extraction yields (%) ± 1 S.D. (n=3). Statistical analysis was performed with Student's t-test. p value: < 0.001 (***) < 0.05 (*). An extraction yield of 100% corresponds to 12.54 mg proteins/mL (2 g cookie in 10 mL buffer).*

As shown in **Figure 35**, increasing the urea concentration significantly improved protein extraction, from 14% without urea to 25% at a concentration equal to or higher than 4 M.

The protein extracts were diluted to 1 mg/mL in 200 mM NH_4HCO_3 and the peptides were analyzed by mass spectrometry. After UHPLC-MS/MS, the peak areas corresponding to the peptides NAVPITLNR, LITLAIPVNKPGR, EAFGVNMQIVR, and GGLEPINFQTAADQAR were found to increase with the urea concentration (**Figure 36**).

The best results were obtained at a urea concentration above 4 M, but the extract was diluted (3 times) to obtain a concentration of 1 mg proteins per mL. Before digestion, the samples were also diluted (1:1 v/v) in 200 mM NH_4HCO_3, so the urea concentration before enzymatic digestion was below 1 M. For better sensitivity, however, it was decided not to dilute the sample to 1 mg/mL prior to digestion. In choosing the final optimized conditions, it was thus necessary to make a compromise

in order to maintain a maximal protein extraction yield without any lost in trypsin activity. At 2 M urea, the yield of the extraction of proteins from cookies was 21%. Before digestion, we chose to dilute the extracts 50:50 v/v in NH_4HCO_3 in order to have a urea concentration lower than 1 M prior to trypsin digestion.

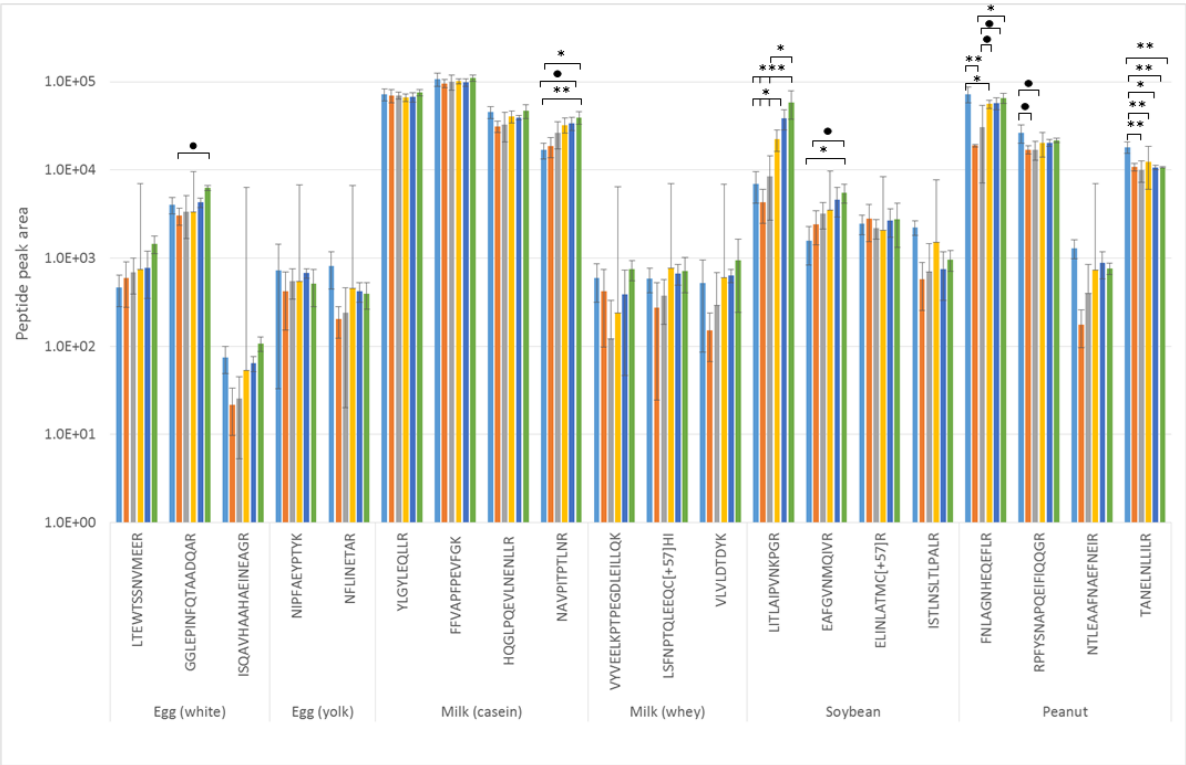

Figure 36: *Effect of the urea concentration on the peak areas of milk, egg, soy, and peanut peptides. Extraction was performed for 30 min with 200 mM Tris-HCl pH 9.2 containing 0 M (light blue), 1 M (orange), 2 M (gray), 3 M (yellow), 4 M (dark blue), or 5 M (green) urea. Results are expressed as mean areas ± 1 S.D. (n=3) and presented on a logarithmic scale. Statistical analysis was performed with Student's t-test. p value: < 0.001 (***) < 0.01 (**) < 0.05 (*) < 0.1 (●).*

I.VIII *Defatting* the cookies

The team of Prof. Martina has compared defatting of heat-processed meat products with hexane, acetone, petroleum ether, and ethanol, and has found acetone to be the most efficient (Castro-Rubio et al., 2005). As cookies contain 16.1% oil, defatting the samples with acetone prior to extraction was tested to determine whether or not this treatment might improve protein extraction. This defatting experiment was performed by analyzing three independent preparations of incurred cookies (180 °C – 18 min) containing 5 g milk, egg, soy, and peanut per kg, with and without flushing with 10 mL acetone.

After digestion, the extracts were directly analyzed by mass spectrometry (**Figure 37**).

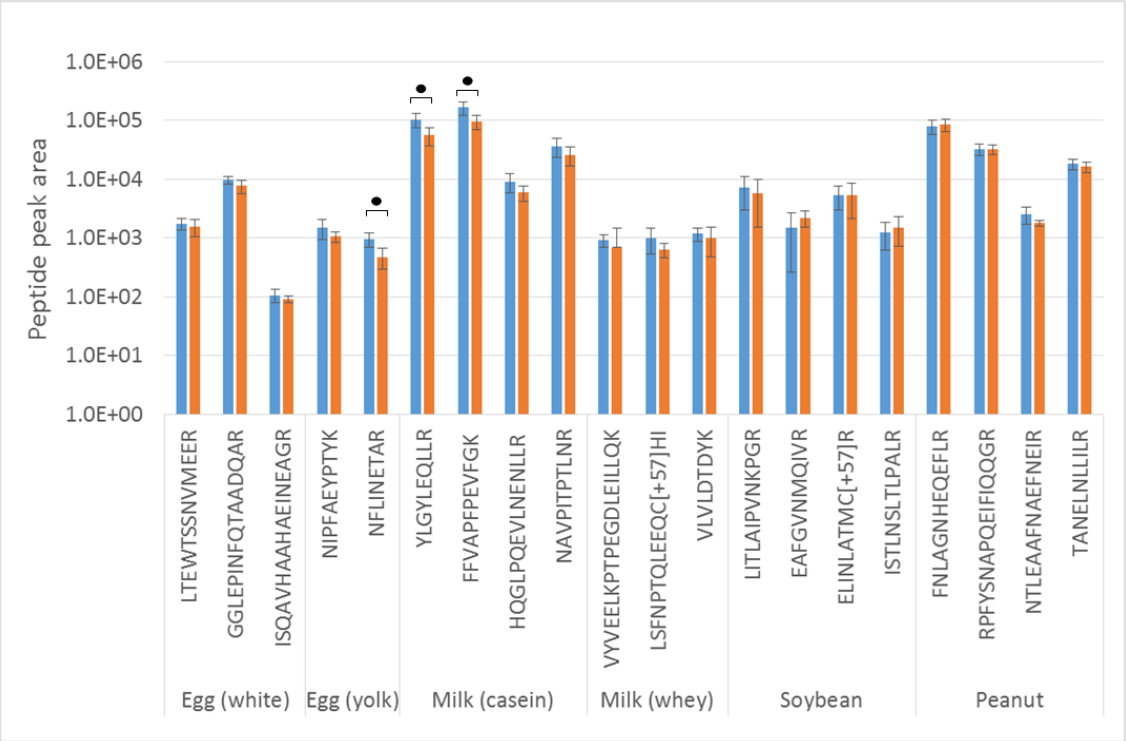

Figure 37: *Effect of defatting the samples on the peak areas corresponding to milk, egg, soy, and peanut peptides extracted with 200 mM TRIS-HCl pH 9.2 containing 2 M urea. Prior to extraction, the samples were washed with acetone (orange) or not (blue) in order to assess the effect of defatting on peptide peak areas. Results are expressed as mean areas ± 1 S.D. (n=3) and presented on a logarithmic scale. Statistical analysis was performed with Student's t-test. p value: < 0.1 (●).*

Statistical analysis revealed slightly significant differences between the defatted and non-defatted extracts for the egg peptide NFLINETAR and the milk peptides YLGYLEQLLR and FFVAPFPEVFGK. In the final protocol, however, we chose not to defat the samples prior to extraction, because defatting did not lead to significantly larger peptide peak areas or cleaner extracts.

I.IX Final selected method for food allergen extraction

Proteins were extracted from 2 g ground matrix in 20 mL extraction buffer (200 mM Tris-HCl pH 9.2, 2 M urea) by shaking at 20 °C for 30 min (Agitelec, France) followed by sonication for 15 min at 4 °C to avoid carbamidomethylation by urea. The samples were centrifuged at 4660 g for 10 min at 10 °C.

The addition of 15 min of ultrasound treatment was based on several studies showing the improvement of protein extraction with sonication. Ultrasounds act principally by generating bubble cavities in the biological matrix that lead to a higher extraction yield (Monaci et al., 2014 b).

II Selection of the purification method

The purification method is a key step in the development of sensitive methods as it is used to concentrate and purify samples before analysis. This step can be applied on proteins or on peptides depending on the method of purification that has been chosen. Two purification methods were considered in this project: solid phase extraction (SPE) for peptide purification and immunoaffinity columns (IAC) for protein purification.

II.I Purification of milk, soy, egg and peanut peptides by SPE

Three independent preparations of incurred cookies (180 °C – 18 min) containing 1350 mg of milk proteins, 3250 mg of egg proteins, 2100 mg of soy proteins, and 1250 mg of peanut proteins per kg of cookies were analyzed with and without purification on a tC18 SPE cartridge. The protocol described in publication (Planque et al., 2016) was followed. The peak areas of milk, egg, soy, and peanut peptides were compared, as shown in **Figure 38**.

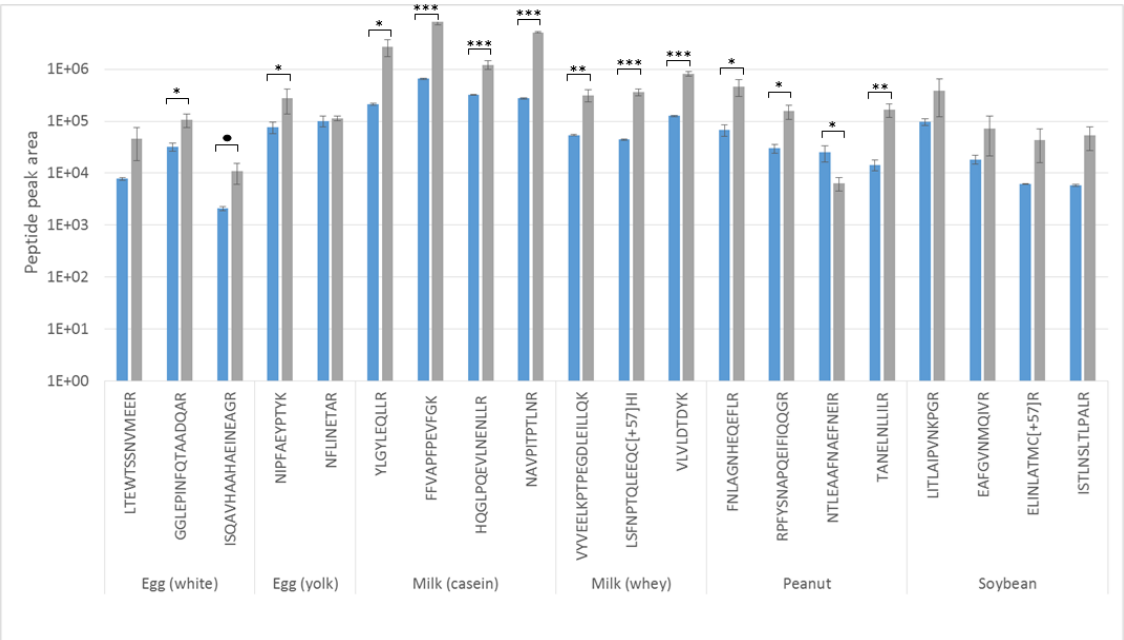

Figure 38: *Effect of SPE purification on the peak areas corresponding to milk, egg, soy, and peanut peptides. Extraction was performed for 30 min with 200 mM Tris-HCl pH 9.2 containing 2 M urea. The samples were analyzed by UHPLC-MS/MS without clean-up (blue) and with tC18 clean-up (gray) to compare the effect of the purification on peptide peak areas. Results are expressed as mean areas ± 1 S.D. (n=3) and presented on a logarithmic scale. Statistical analysis was performed with Student's t-test. p value: < 0.001 (***) < 0.01 (**) < 0.05 (*) < 0.1 (●).*

As tC18 purification was found to improve the peak areas for 14 peptides, this purification step was kept in the protocol.

As the target compounds include peptides with polar and nonpolar properties, two solid phase extraction cartridges were chosen and tested: tC18 (Sep-Pack) (SiC18H37) and HLB (Oasis). The HLB cartridge can retain both nonpolar and polar compounds with its phenyl and pyrrolidone groups, respectively (**Figure 39**), while the tC18 cartridge can only retain nonpolar compounds. Otherwise, the technical specifications/properties (particle size, volume) are quite similar (**Table 4**).

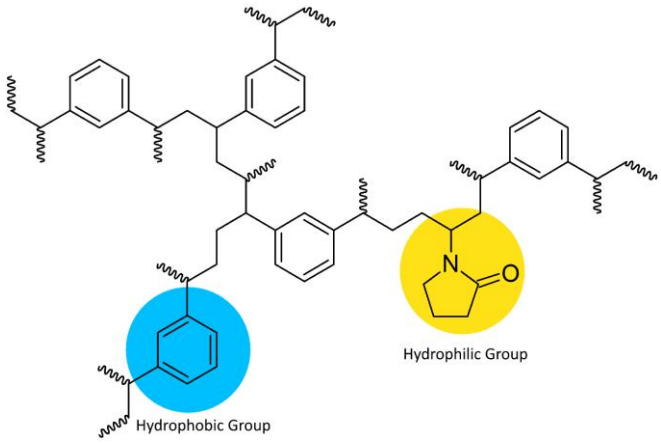

***Figure 39**: The HLB sorbent is a monodisperse N-vinylpyrrolidone-divinylbenzene copolymer resin with a specific mixture of hydrophilic and hydrophobic groups.*

	tC18 (WAT036790 Waters)	**HLB (186000115 Waters)**
Mode	Reversed phase – End Capped	Reversed phase
Sorbent	Silica	Copolymer
Sorbent per cartridge	500 mg	500 mg
Particle size	37 – 55 µm	60 µm
pH range	2 - 8	0 - 14
Pore size	125 Å	80 Å

***Table 4**: Technical specifications of the tC18 (Sep-Pack Waters) and HLB (Oasis Waters) SPE cartridges*

Three independent preparations of incurred cookies (180 °C – 18 min) containing 5 g milk, egg, soy, and peanut per kg were analyzed. The protocol described in publication (Planque et al., 2016) was used to assess the performances of the two cartidges. UHPLC-MS/MS analysis of extracts was performed in order to compare the peptide peak areas obtained with the two SPE columns (**Figure 40**).

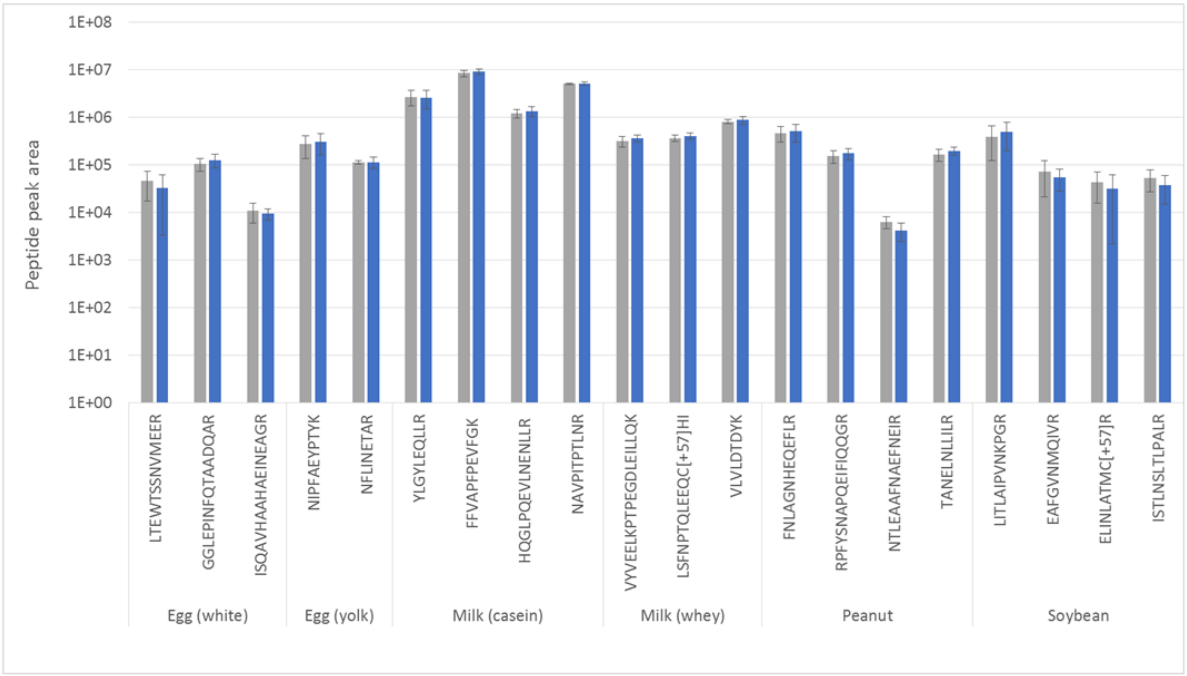

Figure 40 : *Comparison of two SPE cardridges on the peak areas for milk, egg, soy, and peanut peptides. The samples were analyzed by UHPLC-MS/MS with tC18 (blue) or HLB clean-up (gray) to compare the effects of these two purification methods on peptide peak areas. Results are expressed as mean areas ± 1 S.D. (n=3) and presented on a logarithmic scale. Statistical analysis was performed with Student's t-test and no significant difference in peak areas was observed between tC18 and HLB purification.*

The analysis applied to milk, egg, soy, and peanut peptides revealed no significant differences in peptide peak areas obtained with the cartridge used. For this reason, we chose to use the cheaper of the two columns, tC18 SPE.

II.II Purification of milk, soy, egg and peanut peptides with immunoaffinity columns

As purification by solid phase extraction is not protein or peptide specific, nonspecific MRM (Multiple Reaction Monitoring) signals can be observed in complex matrices. Purification with immunoaffinity columns, on the other hand, is highly specific and allows purification of a target protein. This approach, however, could have drawbacks similar to those encountered with ELISAs, i.e. failure of antibodies to recognize processed allergens and bind to them. To avoid such problems, the antibodies used were raised against peptides instead of proteins.

II.II.1 Peptide selection

After the analysis of peptide-containing raw ingredients and processed cookies, several abundant peptides robust to the thermal process were selected. Then, to ensure detection of the corresponding entire proteins by the antibodies, peptide exposure at the surfaces of the native proteins was examined with the open-access software Pymol (**Figure 41**).

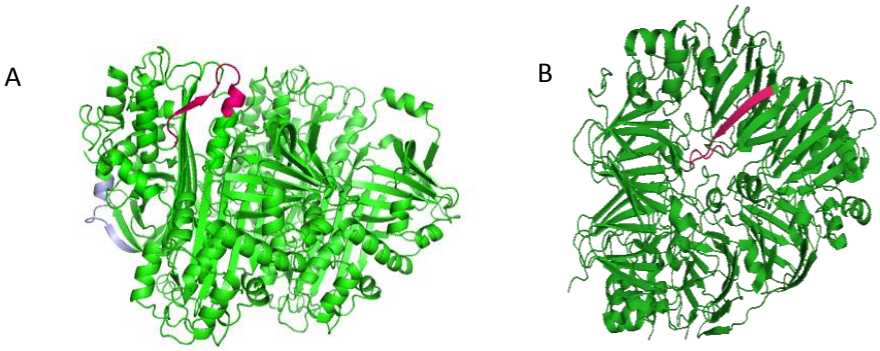

Figure 41*: (A) Structure of native egg ovalbumin (OVA1), obtained with the open-access software Pymol (https://pymol.org/). Two peptides selected for the production of rabbit antibodies are highlighted in pink (GGLEPINFQTAADQAR) and purple (LTEWTSSNVMEER). (B) Structure of native soy glycinin 1 (1UCX), obtained with the open-access software Pymol. The peptide selected for the production of rabbit antibodies is highlighted in pink (ALIQVVNCNGER).*

To elicit an immune response, the peptides (**Table 5**) were synthesized by Eurogentec and coupled with an acyl carrier protein, Keyhole Limpet Hemocyanin (KLH, molecular weight 350 – 400 kDa).

II.II.2 Antibody production

The protocol for rabbit immunization is described in **Figure 42**. For the initial injection, complete Freund's adjuvant (CFA) was added to the antigen solution in order to enhance antibody production. This adjuvant is a water-in-oil emulsion containing heat-killed mycobacteria such as *Mycobacterium tuberculosis* or *Mycobacterium butyricum* (Israeli et al., 2009; Coffman et al., 2010). It acts to prolong the lifetime of the injected antigen and as an immunopotentiator tat can boost antibody production in animal models such as rabbits (Billiau et al., 2001; Fishback et al., 2016). Immunization with protein antigens in CFA stimulates stronger antibody responses and generates marked memory responses, with a mixed T_H1-T_H2 cell phenotype resulting in IFN-γ and IL-4 production (Shibaki et al., 2002; Coffman et al., 2010). For the subsequent injections, Incomplete Freund's Adjuvant (IFA) (which contains no mycobacterial components) mixed with the antigen solution was used to boost antibody production (Jensen et al., 1998; Billiau et al., 2001).

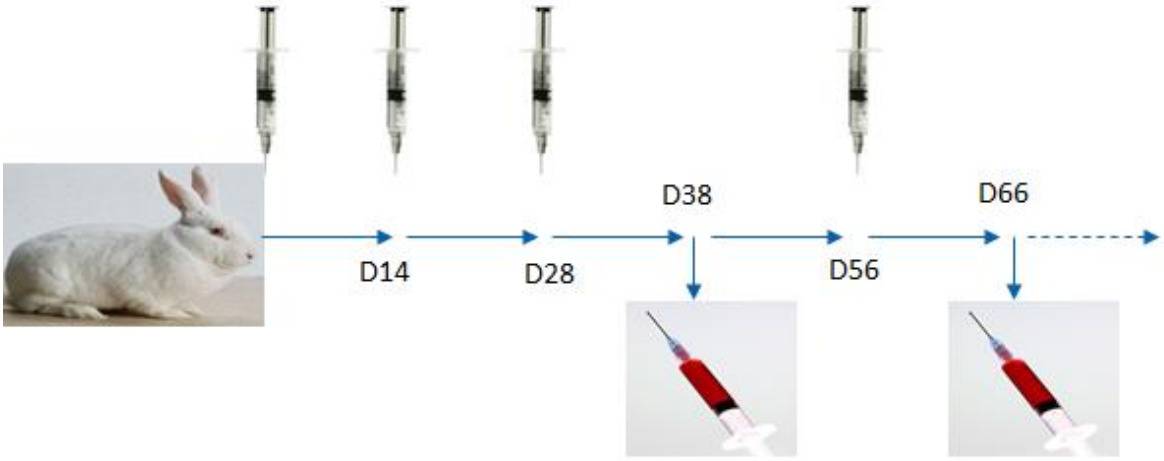

Figure 42: *Injection and serum collection schedule (time in days, D). Rabbits were immunized by subcutaneous injection with 1 mL immunogen emulsified with Freund's complete adjuvant (500 µL saline containing the antigen + 500 µL adjuvant). Freund's incomplete adjuvant was utilized in the subsequent booster injections.The animals received injections containing 0.2 mg antigen. Two-milliliter blood samples were collected from the third immunization onward, 10 days after every boost (Day 38, Day 66…). At the end of the immunization process a final bleeding was done. Samples were centrifuged to eliminate the cell fraction and the sera were stored at –20 °C.*

II.II.3 Recognition of peptides/proteins by ELISA

The ability of the antibodies raised to recognize the relevant antigenic peptide (synthetic peptide or native protein) was assessed by indirect ELISA. Wells were coated with 200 µL peptide or allergen at 1 µg/mL (**Table II-2**). To evaluate the immunogenicity of the antigenic peptide, measurement of antibody titers from rabbit antiseras by indirect ELISA is one of the most important steps. Diluted sera were added to antigen-coated wells and incubated. After washing, an anti-rabbit secondary antibody conjugated with peroxidase was added.

This first test allows to know if an immune response has been triggered and to select the best rabbit sera. The sera must be purified before the development of sandwich ELISA (Miura et al., 2008).

All the antibodies were able to recognize the synthetic peptides, but peptide detection in the native proteins was less obvious. Several antibodies were unable (or weakly able) to recognize the target peptide in the native protein. Milk powder was recognized by 3 of the 4 antibodies raised against casein or β-lactoglobulin peptides.

Whole egg powder (NIST 8445) was not recognized by either of the two antibodies raised against egg white peptides. It was weakly recognized by one egg yolk peptide (DWLVIPDAAAAYIYEAVNK).

Allergen	Protein	Peptide	Recognition of the peptide in the protein
Milk	β-lactoglobulin	VYVEELKPTEGDLEILLQK	YES
		LSFNPTQLEEQCHI	YES
	Casein αs1	FFVAPFPEVFGK	YES
		YLGYLEQLLR	NO
Egg	Ovalbumin	GGLEPINFQTAADQAR	NO
		LTEWTSSNVMEER	NO
	Apovitellenin	NFLINETAR	NO
		DWLVIPDAAAAYIYEAVNK	WEAK
Peanuts	Ara h1	DLAFPGSGEQVEK	YES
		VLLEENAGGEQEER	NO
	Ara h2	RQQWELQGDR	NO
	Aa h3	SPDIYNPQAGSLK	NO
		SQSENFEYVAFK	NO
Soybean	Glycine 1	ALIQVVNCNGER	NO
	Glycine 2	NNNPFSFLPPQESQR	YES
	Beta conglycinin alpha chain	LITLAIPVNKPGR	WEAK
	2S Albumin	QLQGNLTPCEK	WEAK

Table 5: *Sequences of the synthetic peptides used to immunize rabbits and ability of the antibodies to recognize the target allergens.*

Peanut butter (NIST 2387) was recognized by the antibody raised against the Ara h1 peptide (DLAFPGSGEQVEK). A solution containing 10 µg/mL peanut proteins was prepared and heated for 10 min at 100 °C in order to denature the peanut proteins. However, after the denaturation of peanut butter (NIST 2387), Ara h1 protein was not recognized by the antibody.

Soy flour (NIST 3234) was recognized by one antibody out of four raised against glycine 2 peptide (NNNPFSFLPPQESQR).

The promising results obtained with SPE purification and the non-recognition of some native allergens (e.g. egg) by the antibodies raised led us to abandon this strategy in the framework of this project. Indeed, this strategy takes several months for the production of antibodies without any guarentee of promising results for egg and should be investigated as a new project to ensure the detection of proteins in several processed and unprocessed food products . However, the first results constitute encouraging 'proof of concept' of immunization with peptides, because native proteins in 3 allergens were recognized.

Arising from these optimizations, a first study for the detection by UHPLC-MS/MS of milk, egg, soy and peanut marker peptides in processed (cookies and tomato sauce) and unprocessed (chocolate and ice cream) was published (Planque et al., 2016).

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Advances in ultra-high performance liquid chromatography coupled to tandem mass spectrometry for sensitive detection of several food allergens in complex and processed foodstuffs

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ABSTRACT

Sensitive detection of food allergens is affected by food processing and foodstuff complexity. It is therefore a challenge to detect cross-contamination in food production that could endanger an allergic customer's life. Here we used ultra-high performance liquid chromatography coupled to tandem mass spectrometry for simultaneous detection of traces of milk (casein, whey protein), egg (yolk, white), soybean, and peanut allergens in different complex and/or heat-processed foodstuffs. The method is based on a single protocol (extraction, trypsin digestion, and purification) applicable to the different tested foodstuffs: chocolate, ice cream, tomato sauce, and processed cookies. The determined limits of quantitation, expressed in total milk, egg, peanut, or soy proteins (and not soluble proteins) per kilogram of food, are: 0.5 mg/kg for milk (detection of caseins), 5 mg/kg for milk (detection of whey), 2.5 mg/kg for peanut, 5 mg/kg for soy, 3.4 mg/kg for egg (detection of egg white), and 30.8 mg/kg for egg (detection of egg yolk). The main advantage is the ability of the method to detect four major food allergens simultaneously in processed and complex matrices with very high sensitivity and specificity.

1. Introduction

Currently, 220 million people, corresponding to 2–3% of adults and at least 8% of children in the whole human population, suffer from food allergies [1,2]. Ingestion of food allergens can induce formation of allergen-specific immunoglobulin E (IgE) antibodies. A reaction to a food allergen can cause symptoms ranging from light (nausea, vomiting. . .) to severe (e. g. anaphylactic shock). To avoid the risk of adverse reactions, allergens must be totally excluded from the diet of allergic people. For these individuals and the people around them, the need to respect a strict diet results in permanent stress [3–5]. In Europe, 14 food allergen ingredients must appear on labels when present: milk, peanut, egg, soybean, fish, crustaceans, cereals containing gluten, tree nuts, celery, lupin, mustard, sesame, molluscs, and sulfur dioxide [6]. Despite the increasing health problem that food allergies represent and because there are no clear legal food allergen thresholds, Voluntary Incidental Trace Allergen Labeling (VITAL) was developed in New Zealand and Australia to help food producers in the management of cross-contamination during food production. VITAL Reference Doses ED_{01} (milk, egg, and peanuts) and ED_{05} (soybean) are the lower confidence interval for the protection of 99% and 95% of allergenic people, respectively. On the basis of this referential, even though it does not have a regulatory relevance, food product stated on the label to be allergen free should contain less than 2.5 mg milk proteins, 5 mg peanut proteins, 0.75 mg egg proteins, or 25 mg soybean proteins per kilogram (portion size: 40 g) [7–9]. Despite the endangerment of allergic customers, the numerous and various labeling legislations (directives 2007/68/EC, 2000/13/EC and regulation 2011/1169/EC [10–12]), the undeclared presence of food allergens in food products is still widespread [13,14]. To help food producers declare hidden food allergens, several methods have been developed. Among them, the real-time quantitative Reverse Transcription Polymerase Chain Reaction (RT-qPCR) method is based on the detection and analysis of species-specific DNA sequences (coding or not for allergenic proteins), which are more heat stable than proteins [15–17]. Yet PCR cannot discriminate between DNA from egg (allergenic) and chicken meat (non-allergenic), and thus yields false positives or confounding results [18]. Furthermore, because different food allergens have different DNA-to-protein ratios, conversion of amounts

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of DNA to amounts of protein introduces is imprecise and low DNA content can introduce a lack of sensitivity [19]. The most commonly used method is the Enzyme-Linked ImmunoSorbent Assay (ELISA) for routine analysis, but the high similarity of some proteins [20] and structural modifications induced by processing [21–23] frequently lead to false positive or false negative results. For example, thermal processing modifies a protein's structure without necessarily abolishing its allergenic potential [24,25].

Ultra-high performance liquid chromatography coupled to tandem mass spectrometry (UHPLC–MS/MS) for the analysis of food allergens is attracting increasing interest [26–31]. Its specificity and sensitivity make it applicable to both processed and raw food allergens, and it can be used to detect several food allergens simultaneously. In developing a specific and sensitive UHPLC–MS/MS method, several challenges must be met. First, the selected marker peptides must be specific to the target food allergen and robust to the thermal process. Second, the test materials must be incurred and not spiked after the process [32,33]. Third, and especially in the case of processed matrices where protein modifications and degradations might occur [34–36], one must use extraction and purification methods that are adequate for obtaining a Limit Of Quantification (LOQ) of approximately 1 mg/kg, consistent with VITAL recommendations [26,27,29,37]. To meet these challenges and to test the specificity of Multiple Reaction Monitoring (MRM) transitions in allergen-free and contaminated matrices, incurred and processed matrices, selected for their complexity or their thermal process, were prepared. The sensitivity of the method was tested by determining, after ascertainment of linearity, a single, common LOQ for each targeted allergen (milk casein, whey, egg yolk, egg white, soybean, and peanut) in two incurred and two processed matrices: chocolate, ice cream, cookie, and tomato sauce.

2. Materials and methods

2.1. Reagents and materials

Urea, ammonium bicarbonate, tris(hydroxymethyl)aminomethane (TRIS), dimethyl sulfoxide (DMSO), dithiothreitol (DTT), iodoacetamide (IAA), and trypsin from bovine pancreas (T8802) were purchased from Sigma-Aldrich (Bornem, Belgium). Acetonitrile (UHPLC–MS/MS grade) and formic acid were from Biosolve (Valkenswaard, the Netherlands). Acetic acid was obtained from Acros Organics (Geel, Belgium) and hydrochloric acid was from Fisher Chemical (Loughborough, UK). Clean-up was performed with sep-pak tC18 solid phase extraction (SPE) columns, (WAT036790, 6 cc, 500 mg) from Waters (Milford, Massachusetts, USA). Lyophilized milk powder (27% protein) and soy flour (36% protein) were from a local store. Eggs obtained from a local store were used to isolate egg yolk (16% protein) and egg white powder (79% protein) were from Barry Farm Wapakoneta, USA. Peanut butter (NIST 2387 22.2% protein) was from the National Institute of Standards and Technology (NIST) (Gaithersburg, Maryland, USA).

2.2. Marker peptide selection

The *open source* software Skyline was used for in silico enzymatic digestion of food allergen proteins and to design the MRM. The following parameters were selected: peptide length of 8–25 amino acids, b or y fragmentation, carbamidomethylation modification, precursor ion charge 2 or 3 and product ion charge 1 or 2. After in silico digestion, selection of marker peptides in raw food allergens was performed for milk, egg, soy, and peanut prepared individually at 0.1 mg protein/ml. Food proteins were extracted in 200 mM TRIS-HCl; pH 8.2 [32]. Trypsin digestion was carried out (see Section 2.5.2) without any further purification and the digestion products were analyzed by UHPLC–MS/MS. Afterwards, selection of intensely processed peptides was performed in cookies containing milk, egg, soy, and peanut at 5000 mg/kg each, processed for 18 min at 180 °C. Cookie proteins were extracted in 200 mM TRIS-HCl; pH 8.2 with addition of 5 M urea. Samples were then diluted to 1.0 mg protein/ml in 200 mM ammonium bicarbonate, digested with trypsin without any further purification, and analyzed by UHPLC–MS/MS. Finally, after optimization of protein extraction on the basis of protein contents and analysis of peptides (UHPLC–MS/MS), samples of chocolate, ice cream, cookie, and tomato sauce containing proteins from milk, egg, soy, and peanuts at 200 mg/kg were prepared and analyzed according to the sample preparation protocol described below.

2.3. Preparation of non-contaminated matrices

Four target matrices were selected: chocolate, tomato sauce, cookie, and ice cream. Food-allergen-free chocolate, containing mainly cacao (45%), sugar (35%), and rice powder (20%), and tomato sauce composed mainly of tomatoes (75%) and a mixture of onions, carrots, and celery (15%), were purchased from a local shop and finely ground. Ice cream was prepared by combining coco milk (29.6%), sugar (11.0%), lemon juice (0.4%), and banana (59.0%) and mixing these ingredients with a blender. Cookie dough composed of flour (53.4%), sugar (15.2%), water (14.8%), oil (16.1%), salt (0.3%), ammonium bisulfate (0.1%), and sodium bicarbonate (0.1%) was also prepared.

2.4. Preparation of incurred matrices

Incurred matrices were prepared in three independent replicates by adding raw food allergens to non-contaminated matrices to obtain a theoretical allergen protein concentration of 25 mg/kg for milk, 50 mg/kg for soybean, 50 mg/kg for peanut, 100 mg/kg for egg white, and 250 mg/kg for egg yolk. Mixing was carried out with a mechanical blender (robot coupe, Blixer 4 V.V.) for 10 min to ensure homogeneous distribution of food allergens. After incorporation of food allergens, sauce batches were cooked at 95 °C for 45 min, chocolate batches were heated in a water bath at 40 °C for 20 min and frozen at −20 °C before being finely ground, and ice cream was frozen at −20 °C. To obtain incurred matrices with intermediate food allergen concentrations, non-contaminated matrices were mixed with incurred matrices to obtain 0, 0.5, 1.25, 2.5, and 5 mg/kg for milk proteins, 0, 1, 2.5, 5 and 10 mg/kg for soybean and peanut proteins, 0, 3.4, 8.5, 17, 34 mg/kg for egg proteins (egg white), and 0, 12.3, 30.8, 61.6, 123.2 mg/kg for egg proteins (egg yolk). Afterwards, each cookie (40 g dough with a diameter of 7 cm) was baked at 180 °C for 18 min and finely ground.

2.5. Sample preparation protocol

2.5.1. Extraction

Proteins were extracted from 2 g ground matrix in 20 ml of 200 mM TRIS-HCl, pH 9.2, 2 M urea by shaking at 20 °C for 30 min (Agitelec, France) followed by sonication for 15 min at 4 °C to avoid carbamidomethylation with urea. The samples were centrifuged at 4660*g* for 10 min at 10 °C.

2.5.2. Digestion

A 10-ml aliquot of supernatant was diluted in 10 ml of 200 mM ammonium bicarbonate. After addition of 1 ml of 200 mM DTT to reduce proteins, the samples were incubated at 20 °C for 45 min. Subsequently, alkylation of these proteins was performed by adding 2 ml of 500 mM IAA and incubating for 45 min at 20 °C in the dark. A 16-h trypsin digestion (1 mg/ml in 50 mM acetic acid)

Table 1
Number of proteins, peptides and transitions for milk, egg, soybean and peanut allergens, according five criteria, for the final selection of marker peptides.

		Milk		Egg		Peanuts	Soybeans	Total
		Caseins	Whey proteins	White	Yolk			
1	Proteins	4	3	5	3	43	25	83
Uniprot								
2	Peptides	29	48	63	120	335	345	940
Skyline In-silico digestion	Transitions	648	969	1278	2473	7137	7419	19924
3	Peptides	14	19	41	46	61	44	225
Raw ingredients analysis	Transitions	62	92	197	214	329	179	1073
4	Peptides	11	5	12	11	15	26	80
Processed cookies 5000 ppm	Transitions	39	18	43	38	56	119	313
5	Peptides	4	3	3	5	4	4	23
Incurred and processed matrices	Transitions	12	9	9	15	12	12	69

was performed (protein:trypsin ratio1:20) at 37 °C. The reaction was stopped by addition of 300 μl of 20% formic acid.

2.5.3. SPE purification

Digested proteins were purified on tC18 SPE columns. Cartridge pre-conditioning was performed with 18 ml acetonitrile followed by equilibration with 18 ml of 0.1% formic acid. The digested proteins were centrifuged at 4660*g* for 5 min at 10 °C and 20 ml supernatant was loaded on the column. Next, 18 ml of 0.1% formic acid was used to flush out impurities. Elution was then performed with 1.8 ml of acetonitrile/0.1% formic acid 30/70 v/v followed by 3.6 ml acetonitrile/0.1% formic acid 80/20: v/v. Before evaporation at 40 °C under a nitrogen flow, 30 μl DMSO was added to avoid dryness. After evaporation, the pellets were dissolved in 200 μl of 0.1% formic acid and centrifuged for 5 min at 11754*g*. Peptides were then analyzed by UHPLC–MS/MS.

2.5.4. UHPLC–MS/MS

An Acquity system (Milford, Massachusetts, USA) equipped with a C18 Acquity BEH130 Waters column (2.1 × 150 mm) was used to separate the food allergen peptides. Column compartment and thermal autosampler were set at 40 °C and 10 ± 5 °C, respectively. After injection of 20 μl sample, a gradient applied for 24 min (at 0.2 ml/min) allowed separation of the food allergen peptides (solvent: 0.1% formic acid (A) – acetonitrile plus 0.1% formic acid (B)). Elution was carried out as follows: 0–1 min: 86% A; 1–16.5 min: 86% to 60% A, 16.5–16.6 min: 60% to 0% A; 16.6–21 min: 0% A; 21.0–21.1 min: 0% to 86% A, 21.1–24 min: 86% A. A Waters Xevo TQS triple quadrupole system with a positive electrospray and MRM mode were used for detection of food allergen peptides. A 150 l/h cone flow and a 1200 l/h desolvation flow of nitrogen were then applied. The capillary voltage was set at 2.0 kV and the collision gas flow was set at 0.12 ml/min. The source and desolvation temperatures were set respectively at 150 and 500 °C.

3. Results and discussion

3.1. Marker peptide selection

Marker peptides were selected in five steps (Table 1). First, the Uniprot database was searched for target proteins of milk, egg, soy, and peanut. Second, peptides and transitions were generated by Skyline in silico digestion. Third, digested extracts of raw food allergens were analyzed by UHPLC–MS/MS, without prior purification. This allowed elimination of 95% of the 19924 transitions generated by Skyline. Fourth, processed cookies were analyzed to identify a list of peptides resistant to the thermal process and having intense transitions as in the analysis of raw ingredients. Fifth, the target food allergens were incorporated into the chocolate, ice cream, sauce, and cookies and extracted, digested with trypsin, and purified on SPE columns. Marker peptides were determined by UHPLC–MS/MS in all target matrices and only specific peptides giving high-intensity transitions and detected in all matrices were retained (Table 2). Even through UHPLC–MS/MS method is highly specific and peptide specificity was controlled (blast), the huge number of proteins in complex foodstuffs can lead to unspecific transitions. Moreover, structural protein modifications and interferences in complex foodstuffs can also compromise food allergen detection. For all these reasons, the detection of one peptide per allergen is not enough and it is therefore essential to ensure the specificity and the reliability of the method by keeping the detection of at least 3 or 4 peptides for each allergen. Collision energies were optimized for each peptide transition with a 0.1 mg/ml solution of raw ingredient proteins. The three most intense transitions for each peptide were retained for subsequent analyses.

3.2. Determination of marker peptide specificity

A BLAST analysis of the retained peptide sequences was performed to check for inter-species homology (Uniprot). The egg peptides ISQAVHAAHAEINEAGR and EALQPIHDLADEAISR were found in both hen eggs and *Coturnix japonica* (common farmed quail) eggs. The peptides NIPFAEYPTYK and NIGELGVEK of hen eggs displayed homology to peptides of *Anas platyrhynchos* (mallard duck) eggs. As the production and price of hen eggs totally exclude eggs from other species in industrial preparations, these similarities were not viewed as a drawback of the method. The milk proteins of bovine, ovine, and caprine species tend to show high homology. For instance, casein αS1 of *dairy cow* milk shares 95.8% similarity to that of *Bubalus bubalis* (buffalo), 99.1% similarity to that of *Bos mutus grunniens* (yak), 88.8% similarity to that of *Ovis aries* (sheep), and 88.3% similarity to that of *Capra hircus* (goat) (Uniprot). However, most people who are allergic to cow milk are also allergic to the milk of these other animals [20]. Therefore, the peptides YLGYLEQLLR and VLVLDTDYK, homologous to peptides found in the milk of the above-mentioned species, were retained in the final selection. No homologies relevant to food ingredients were found for the selected soy and peanut peptides.

3.3. Method sensitivity

For each food allergen, a single, common LOQ was determined for all targeted matrices (Fig. 1 A and B). For each peptide, two MRM transitions in allergen-free matrices and in incurred matrices are shown to demonstrate the specificity of the method and to confirm detection of the food allergens at the LOQ. The retention times of peptides were different between matrices. Indeed, matrix effect can affect detection of food allergen peptides and might lead to some shift in the retention time. The introduction of an internal standard will allow to determine relative retention time and to limit the matrix effects. The LOQ was defined as the minimum concentration giving a signal-to-noise ratio (S/N) of 10 for the most intense MRM

Table 2
Multiple Reaction Monitoring (MRM) parameters for the identification of milk, egg, soybean and peanut proteins by UHPLC–MS/MS. The cone voltage was fixed at 35 V.

	Protein	Peptide	Retention time in sauce (min)	Precursor (charge state) (*m*/*z*)	Product ion (fragment)	Collision energy (eV)
Soybean	Glycinin P04347 *Glycinin A3B4*	ISTLNSLTLPALR	10.5	699.9 (++)	984.6 ($y9^+$) 870.5 ($y8^+$) 783.5 ($y7^+$)	23 25 25
	Glycinin G2 P04405 *Gly m 6*	EAFGVNMQIVR	8.1	632.3 (++)	859.5 ($y7^+$) 760.4 ($y6^+$) 646.4 ($y5^+$)	18 17 22
	2S albumin P19594 *Gly m 2S albumin*	ELINLATMCR	8.3	610.8 (++)	865.4 ($y7^+$) 751.4 ($y6^+$) 638.3 ($y5^+$	21 21 17
	Beta-conglycinin P13916 *Gly m Bd60 K*	LITLAIPVN**K**PGR	7.9	464.6 (+++)	767.5 ($y7^+$) 583.4 ($y11^{++}$) 476.3 ($y9^{++}$)	15 9 11
Milk	Casein αS1 P02662 *Bos d 8*	HQGLPQEVLNENLLR	8.1	587.3 (+++)	871.5 ($y7^+$) 758.4 ($y6^+$) 436.2 ($b4^+$)	17 16 17
		FFVAPFPEVFGK	13.5	692.9 (++)	991.5 ($y9^+$) 920.5 ($y8^+$) 676.4 ($y6^+$)	18 18 28
		YLGYLEQLLR	12.3	634.4 (++)	934.5 ($y7^+$) 771.5 ($y6^+$) 658.4 ($y5^+$)	21 20 21
	Casein αS2 P02663	NAVPITPTLNR	5.1	598.3 (++)	911.5 ($y8^+$) 456.3 ($y8^{++}$) 285.2 ($b3^+$)	17 14 12
	β-lactoglobulin P02754 *Bos d 5*	VYVEEL**K**PTPEGDLEILLQK	10.6	771.8 (+++)	912.0 ($y16^{++}$) 790.9 ($y14^{++}$) 627.9 ($y11^{++}$)	19 19 20
		VLVLDTDYK	6.4	533.3 (++)	853.4 ($y7^+$) 754.4 ($y6^+$) 641.3 ($y5^+$)	15 14 16
		LSFNPTQLEEQCH*IN*-terminal peptide	8.9	858.4 (++)	1254.6 ($y10^+$) 928.4 ($y7^+$) 627.8 ($y10^{++}$)	26 27 27
Egg	Ovalbumin P01012 *Gal d 2*	GGLEPINFQTAADQAR	7.5	844.4 (++)	1331.7 ($y12^+$) 1121.5 ($y10^+$) 666.3 ($y12^+$)	26 28 25
		LTEWTSSNVMEER	5.9	791.4 (++)	1052.5 ($y9^+$) 951.4 ($y8^+$) 864.4 ($y7^+$)	31 23 23
		ISQAVHAAHAEINEAGR	2.3	887.5 (++)	1138.6 ($y11^+$) 1067.5 ($y10^+$) 996.5 ($y9^+$)	33 33 32
	Vitellogenin-2 P02845	EALQPIHDLADEAISR	7.8	593.3 (+++)	761.4 ($y7^+$) 690.3 ($y6^+$) 668.8 ($y12^{++}$)	19 15 15
		NIPFAEYPTYK	7.5	671.8 (++)	1115.5 ($y9^+$) 508.3 ($y4^+$) 558.3 ($y9^{++}$)	15 16 29
		NIGELGVEK	4.0	479.8 (++)	731.4 ($y7^+$) 674.4 ($y6^+$) 545.3 ($y5^+$)	12 10 19
	Vitellogenin-1 P87498	YLLDLLPAAASHR	10.4	480.6 (+++)	709.4 ($y7^+$) 582.3 ($y11^{++}$) 355.2 ($y7^{++}$)	15 10 14
	Apovitellenin P02659	NFLINETAR	6.2	539.3 (++)	816.5 ($y7^+$) 703.4 ($y6^+$) 590.3 ($y5^+$)	14 16 16
Peanut	Cupin P43238 Ara h1	NTLEAAFNAEFNEIR	10.7	869.9 (++)	1139.5 ($y9^+$) 992.5 ($y8^+$) 878.4 ($y7^+$)	27 26 26
	Cupin Q8LKN1 Ara h 3/4	RPFYSNAPQEIFIQQGR	7.3	684.4 (+++)	748.4 ($y6^+$) 608.3 ($y10^{++}$) 836.4 ($b7^+$)	20 19 17
		FNLAGNHEQEFLR	6.2	525.6 (+++)	692.4 ($y5^+$) 600.8 ($y10^{++}$) 565.3 ($y9^{++}$)	20 13 14
	Cupin Q647H4 Ahy-1	TANELNLLILR	11.2	635.4 (++)	983.6 ($y8^+$) 854.6 ($y7^+$) 741.5 ($y6^+$)	21 20 22

transition of the targeted food allergen. The S/N was calculated peak to peak in a range equal to six times the peak width at half height for three independent replicates of the targeted matrix. The results obtained demonstrate the suitability of the method. The sensitivity of detection for the food allergen peptides was determined on processed cookies. The LOQs recorded are: 0.5 mg milk proteins/kg for caseins, 5 mg milk proteins/kg for whey, 3.4 mg egg proteins/kg for egg white, 30.8 mg egg proteins/kg for egg yolk, 2.5 mg/kg for peanut proteins, and 5 mg/kg for soybean proteins. On the basis of the LOQ, milk casein and egg white peptides appear better for detecting food allergens than whey and egg yolk proteins, as the sensitivity of detection is ten times higher for these peptides than for whey and egg yolk peptides. In industrial processes, however, whey and egg yolk can easily be separated, respectively, from milk casein and egg white. It is therefore important to have a sensitive

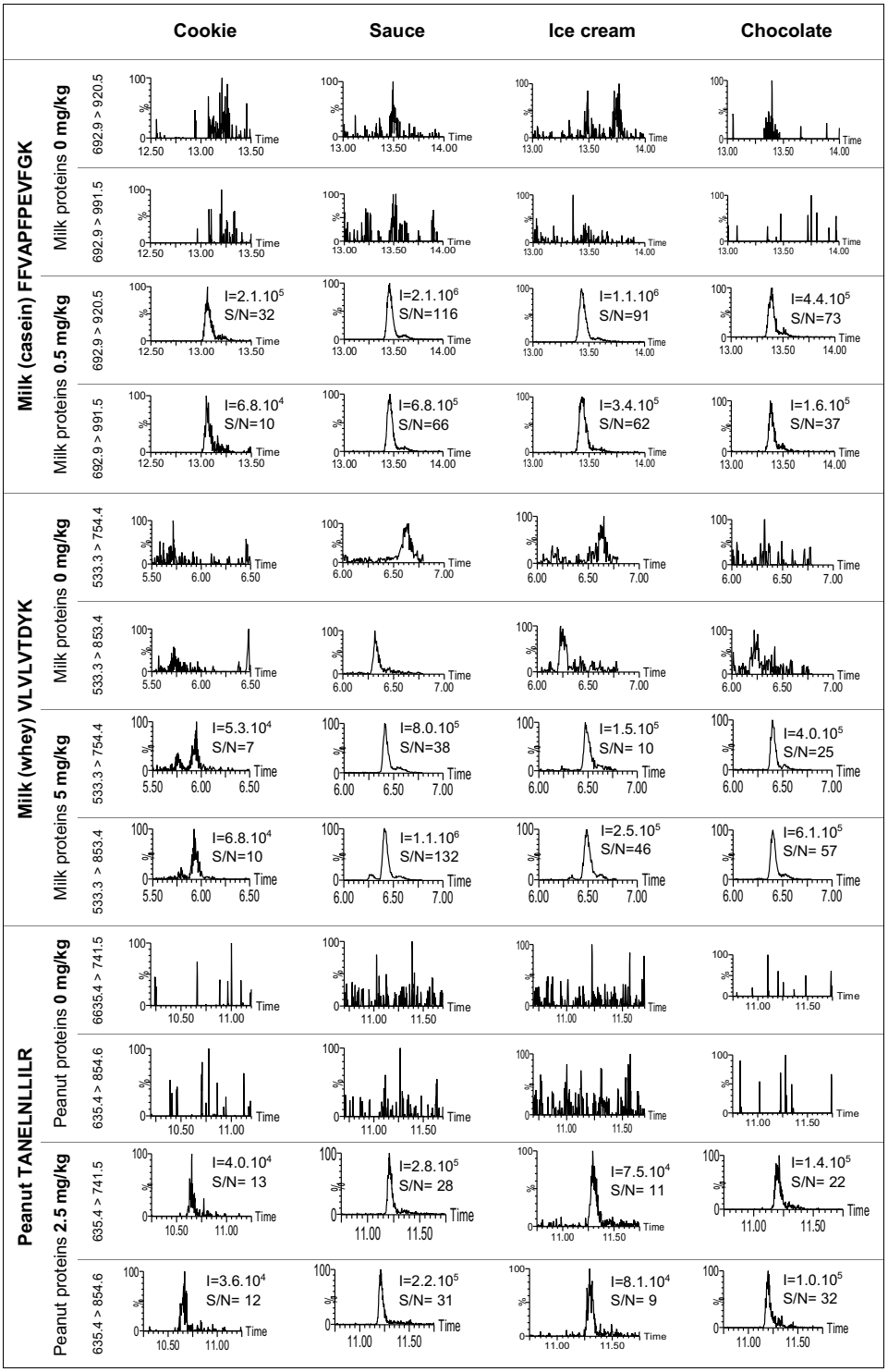

Fig. 1. A and B: Chromatograms of the two higher multiple reaction monitoring MRM transtion of 1-A; milk casein peptide FFVAPFPEVFGK, whey milk peptide VLVLDTDYK and peanut peptide TANELNLLILR 1-B: egg white peptide GGLEPINFQTAADQAR, egg yolk peptide NFLINETAR and soy peptide EAFGVNMQIVR in chocolate, ice cream, tomato sauce and cookies. Data of non-contaminated matrices (0 mg/kg), incurred or processed matrices at the limit of quantification are presented without any data treatment.

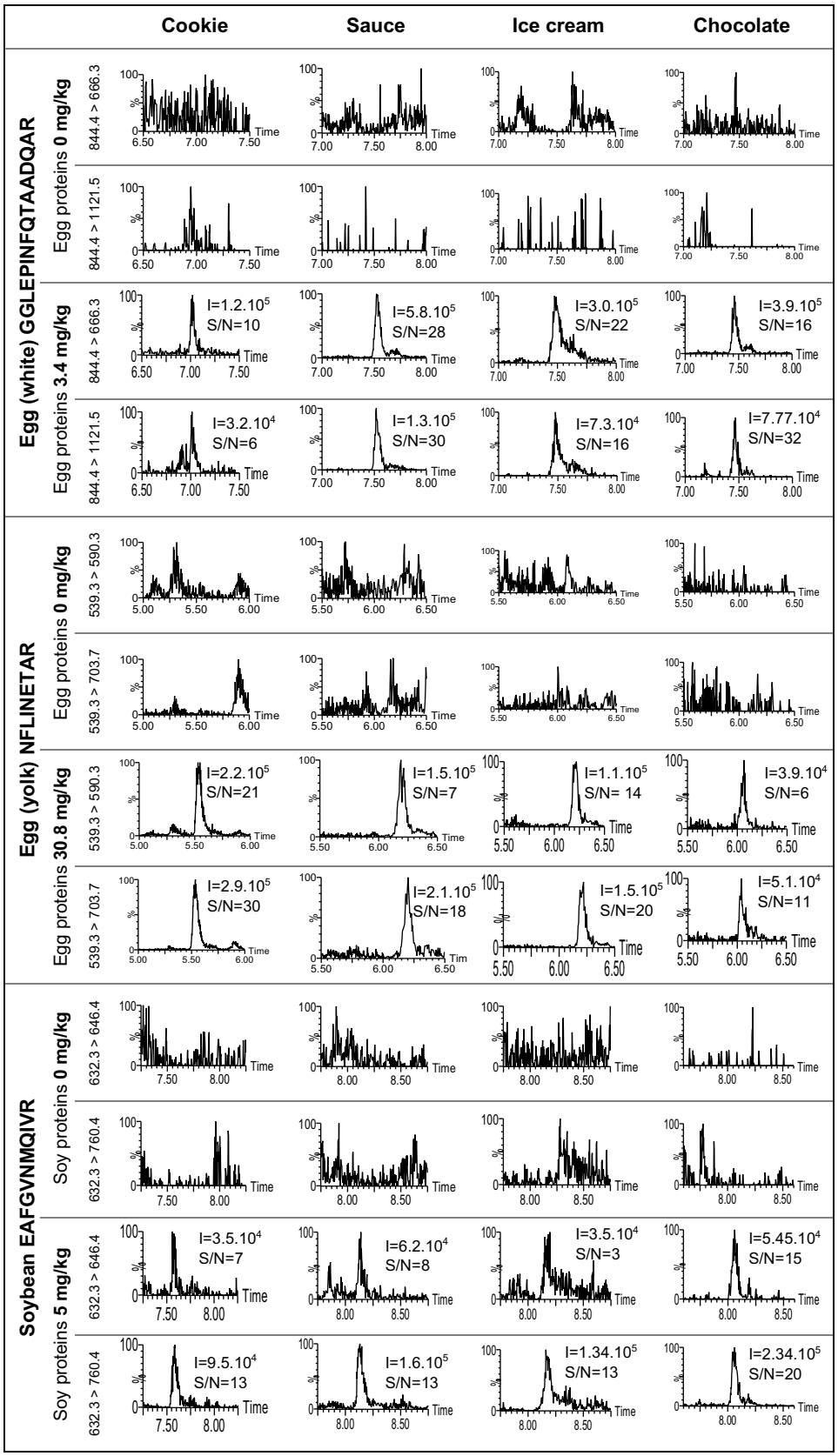

Fig. 1. (*Continued*)

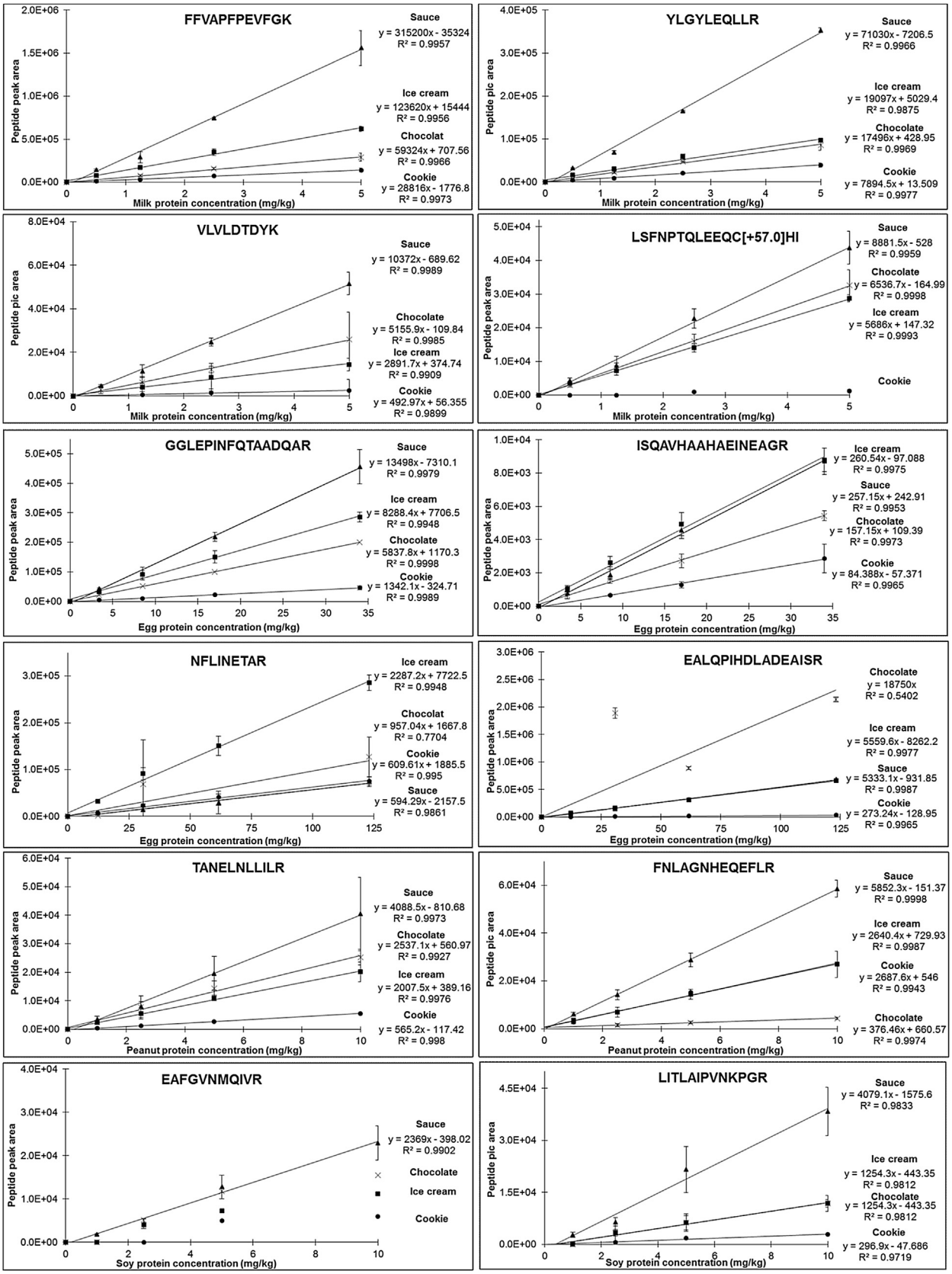

Fig. 2. Linear regression of peptide peak area of the higher MRM in function of the concentration of food allergen proteins performed in three independent replicates in incurred tomato sauce, chocolate, ice cream and processed cookies. The linearity was controlled for each food allergen: milk casein FFVAPFPEVFGK (692.9 > 920.5) and YLGYLEQLLR (634.4 > 771.5), whey milk VLVLDTDYK (533.3 > 853.4) and LSFNPTQLEEQC[+57]HI (858.4 > 928.4) (carbamidomethylation of cysteine amino acids by addition of iodoacetamide before an enzymatic digestion to block the onset of disulfur bridges), egg white GGLEPINFQTAADQAR (844.4 > 666.3) and ISQAVHAAHAEINEAGR (887.5 > 1067.5), egg yolk NFLINETAR (539.3 > 703.4) and EALQPIHDLADEAISR (593.3 > 668.8), peanut TANELNLLILR (635.4 > 741.5) and FNLAGNHEQEFLR (525.6 > 600.8), soybean EAFGVNMQIVR (632.9 > 760.4) and LITAIPVNKPGR (464.6 >583.4)".

detection of egg yolk and whey peptides because of their allergenic properties.

3.4. Matrix effects

Linearity and matrix effects were tested by analyzing three independent foodstuff preparations (incurred chocolate and ice cream and processed cookies and sauce) containing different concentrations of milk, egg, soy, and peanut food allergen proteins (Fig. 2). For VLVLDTDYK and EAFGVNMQIVR in chocolate, cookies, and ice cream, it was not possible to draw a linear regression curve regarding to the LOQs. As shown in Fig. 2, the matrix effect and the effect of the thermal process were not the same for both targeted peptides from the same food allergen. For example, heating for 45 min at 95 °C was found to affect the ovalbumin peptide GGLEPINFQTAADQAR more weakly than the apovitellenin peptide NFLINETAR. Yet no matter how the matrix and thermal effects varied, the linear coefficient of regression supported the reliability of the method even the absence of an internal standard, except for the NFLINETAR peptide in chocolate ($R^2 = 0.77$) and EALQPIHDLADEAISR ($R^2 = 0.54$) from egg yolk proteins. As these egg yolk peptides can be detected with sufficient specificity and sensitivity in processed and incurred matrices, in future egg yolk protein quantifications one should mitigate the lack of linearity by introducing internal standards.

4. Conclusion

With a view to improving food allergen labeling and ultimately the quality of life of allergic people, a sensitive qualitative multi-allergen detection method was developed for two incurred and two –processed matrices. Sensitive detection of food allergens (milk casein, whey, egg white, egg yolk, peanut, and soybean) was achieved by analyzing food allergen peptides by ultra-high performance liquid chromatography coupled to tandem mass spectrometry (UHPLC–MS/MS). In keeping with food production requirements, the targeted matrices were processed (tomato sauce, cookies) or incurred (chocolate, ice cream). The sensitivity of the method was determined on the basis of the signal-to-noise ratio. To our knowledge, this study is the first to identify a such high sensitivity whey and egg yolk marker peptides by UHPLC–MS/MS with very high sensitivity in both processed and incurred material. Our multi-allergen detection method has the lowest limits of quantification available to date (expressed in total proteins and not soluble proteins): 0.5 mg milk proteins/kg for caseins, 5 mg milk proteins/kg for whey, 3.4 mg egg proteins/kg for egg white, 30.8 mg egg proteins/kg for egg yolk, 2.5 mg peanut proteins/kg and 5 mg soybean proteins/kg. The method shows an LOQ below the VITAL threshold (portion size: 40 g) for milk casein, peanut, and soybean, but values 2, 4.5, and 45 times the VITAL threshold for whey milk, egg white, and egg yolk, respectively. With a view to developing a quantitative method, the introduction of internal standards and extension to other allergens are under investigation.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chroma.2016.08.033.

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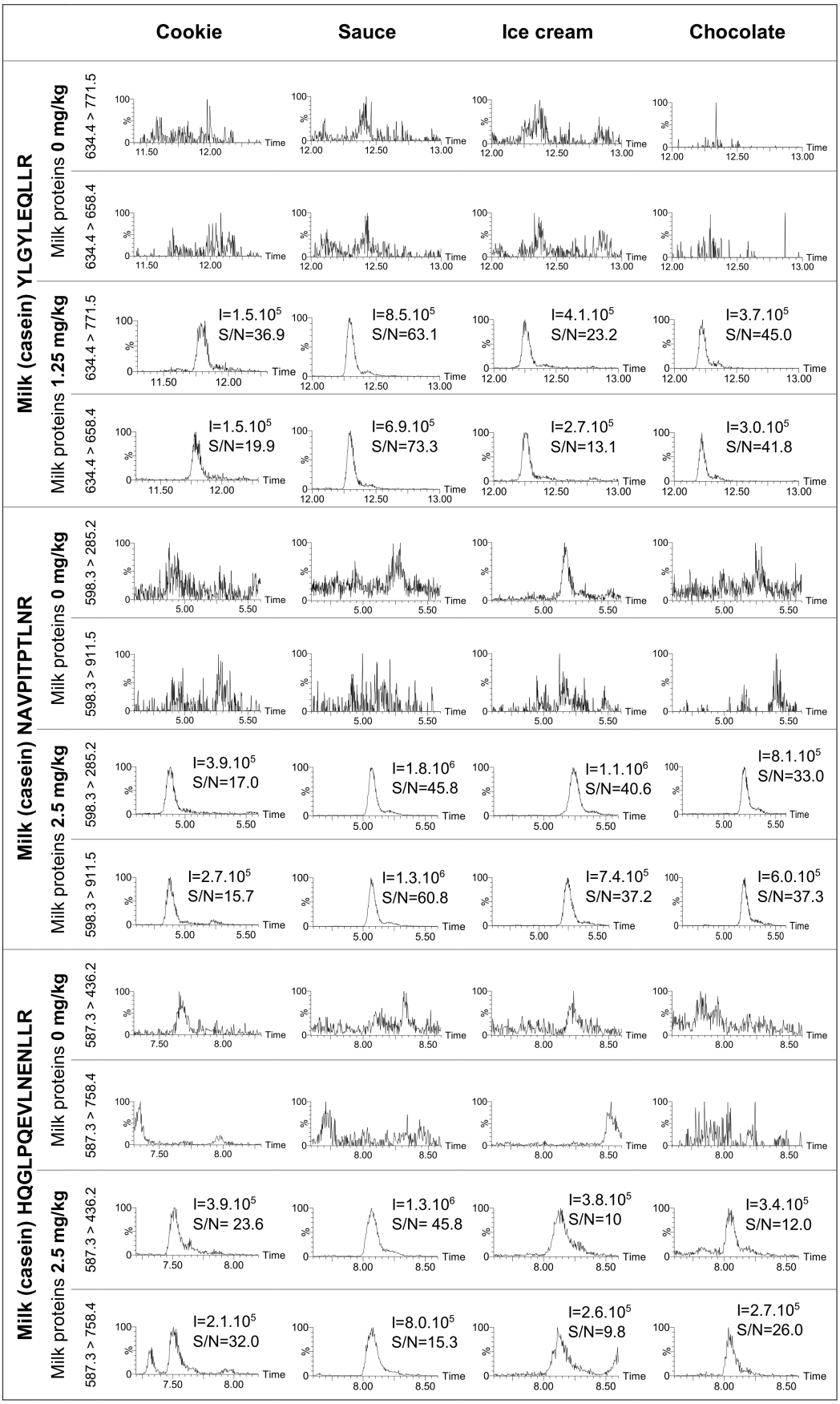
Supplementary data - Figure 1: Chromatograms of the two higher multiple reaction monitoring MRM transtion of egg, milk, soy and peanut allergens. Data of non-contaminated matrices (0 mg/kg), incurred or processed matrices at the limit of quantification are presented without any data treatment.


Cookie
Sauce
Ice cream
Chocolate
Milk (casein) YLGYLEQLLR
Milk proteins 0 mg/kg
634.4 > 771.5
634.4 > 658.4
Milk proteins 1.25 mg/kg
634.4 > 771.5
I=1.5.10^5 S/N=36.9
I=8.5.10^5 S/N=63.1
I=4.1.10^5 S/N=23.2
I=3.7.10^5 S/N=45.0
634.4 > 658.4
I=1.5.10^5 S/N=19.9
I=6.9.10^5 S/N=73.3
I=2.7.10^5 S/N=13.1
I=3.0.10^5 S/N=41.8
Milk (casein) NAVPITPTLNR
Milk proteins 0 mg/kg
598.3 > 285.2
598.3 > 911.5
Milk proteins 2.5 mg/kg
598.3 > 285.2
I=3.9.10^5 S/N=17.0
I=1.8.10^6 S/N=45.8
I=1.1.10^6 S/N=40.6
I=8.1.10^5 S/N=33.0
598.3 > 911.5
I=2.7.10^5 S/N=15.7
I=1.3.10^6 S/N=60.8
I=7.4.10^5 S/N=37.2
I=6.0.10^5 S/N=37.3
Milk (casein) HQGLPQEVLNENLLR
Milk proteins 0 mg/kg
587.3 > 436.2
587.3 > 758.4
Milk proteins 2.5 mg/kg
587.3 > 436.2
I=3.9.10^5 S/N= 23.6
I=1.3.10^6 S/N= 45.8
I=3.8.10^5 S/N=10
I=3.4.10^5 S/N=12.0
587.3 > 758.4
I=2.1.10^5 S/N=32.0
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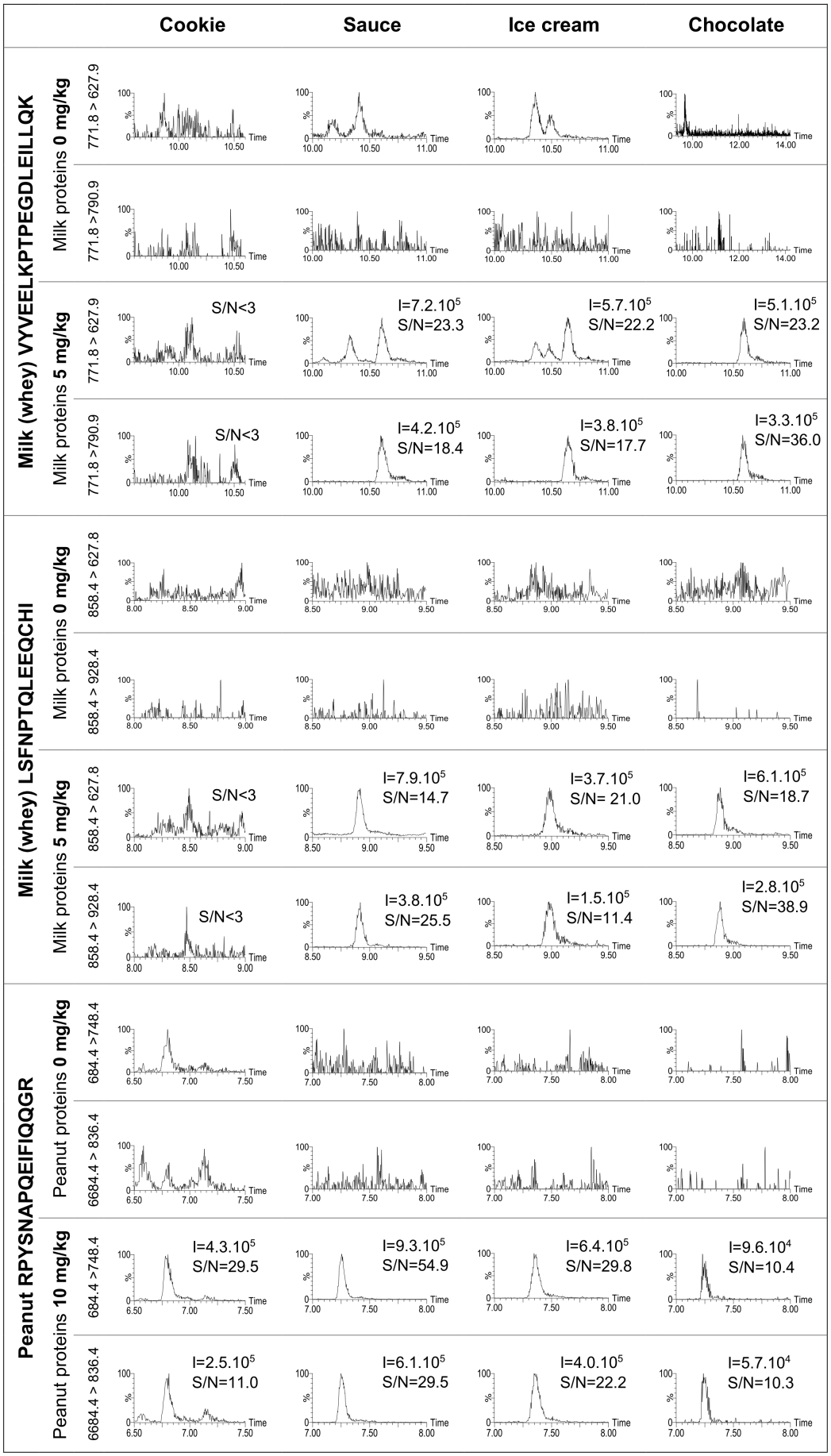
Cookie
Sauce
Ice cream
Chocolate
Milk (whey) VYVEELKPTPEGDLEILLQK
Milk proteins 0 mg/kg
771.8 > 627.9
771.8 >790.9
Milk proteins 5 mg/kg
771.8 > 627.9
771.8 >790.9
S/N<3
I=7.2.10^5 S/N=23.3
I=5.7.10^5 S/N=22.2
I=5.1.10^5 S/N=23.2
S/N<3
I=4.2.10^5 S/N=18.4
I=3.8.10^5 S/N=17.7
I=3.3.10^5 S/N=36.0
Milk (whey) LSFNPTQLEEQCHI
Milk proteins 0 mg/kg
858.4 > 627.8
858.4 > 928.4
Milk proteins 5 mg/kg
858.4 > 627.8
858.4 > 928.4
S/N<3
I=7.9.10^5 S/N=14.7
I=3.7.10^5 S/N= 21.0
I=6.1.10^5 S/N=18.7
S/N<3
I=3.8.10^5 S/N=25.5
I=1.5.10^5 S/N=11.4
I=2.8.10^5 S/N=38.9
Peanut RPYSNAPQEIFIQQGR
Peanut proteins 0 mg/kg
684.4 >748.4
6684.4 > 836.4
Peanut proteins 10 mg/kg
684.4 >748.4
6684.4 > 836.4
I=4.3.10^5 S/N=29.5
I=9.3.10^5 S/N=54.9
I=6.4.10^5 S/N=29.8
I=9.6.10^4 S/N=10.4
I=2.5.10^5 S/N=11.0
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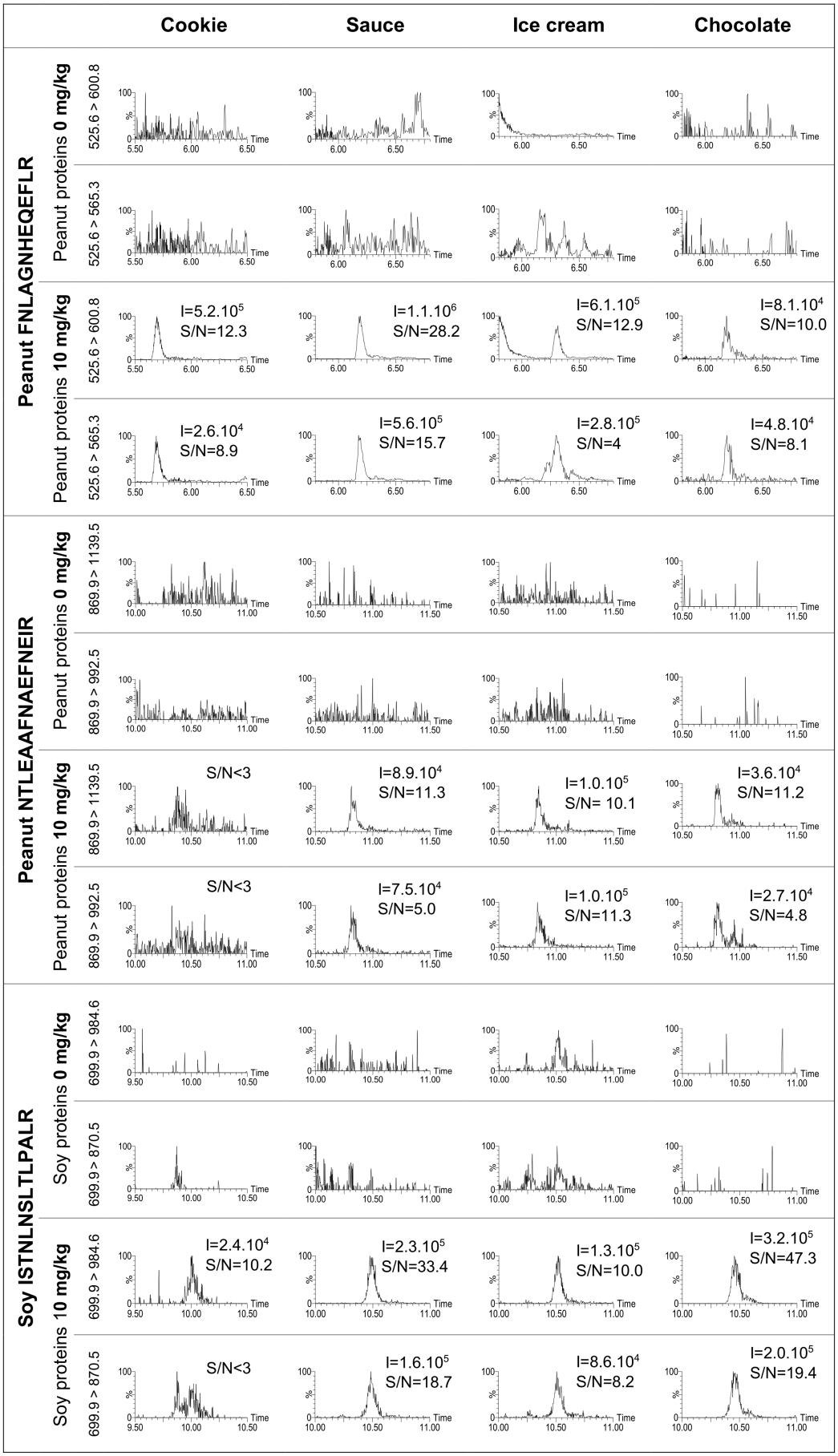
Cookie
Sauce
Ice cream
Chocolate
Peanut FNLAGNHEQEFLR
Peanut proteins 0 mg/kg
525.6 > 600.8
525.6 > 565.3
Peanut proteins 10 mg/kg
525.6 > 600.8
I=5.2.10^5 S/N=12.3
I=1.1.10^6 S/N=28.2
I=6.1.10^5 S/N=12.9
I=8.1.10^4 S/N=10.0
525.6 > 565.3
I=2.6.10^4 S/N=8.9
I=5.6.10^5 S/N=15.7
I=2.8.10^5 S/N=4
I=4.8.10^4 S/N=8.1
Peanut NTLEAAFNAEFNEIR
Peanut proteins 0 mg/kg
869.9 > 1139.5
869.9 > 992.5
Peanut proteins 10 mg/kg
869.9 > 1139.5
S/N<3
I=8.9.10^4 S/N=11.3
I=1.0.10^5 S/N= 10.1
I=3.6.10^4 S/N=11.2
869.9 > 992.5
S/N<3
I=7.5.10^4 S/N=5.0
I=1.0.10^5 S/N=11.3
I=2.7.10^4 S/N=4.8
Soy ISTNLNSLTLPALR
Soy proteins 0 mg/kg
699.9 > 984.6
699.9 > 870.5
Soy proteins 10 mg/kg
699.9 > 984.6
I=2.4.10^4 S/N=10.2
I=2.3.10^5 S/N=33.4
I=1.3.10^5 S/N=10.0
I=3.2.10^5 S/N=47.3
699.9 > 870.5
S/N<3
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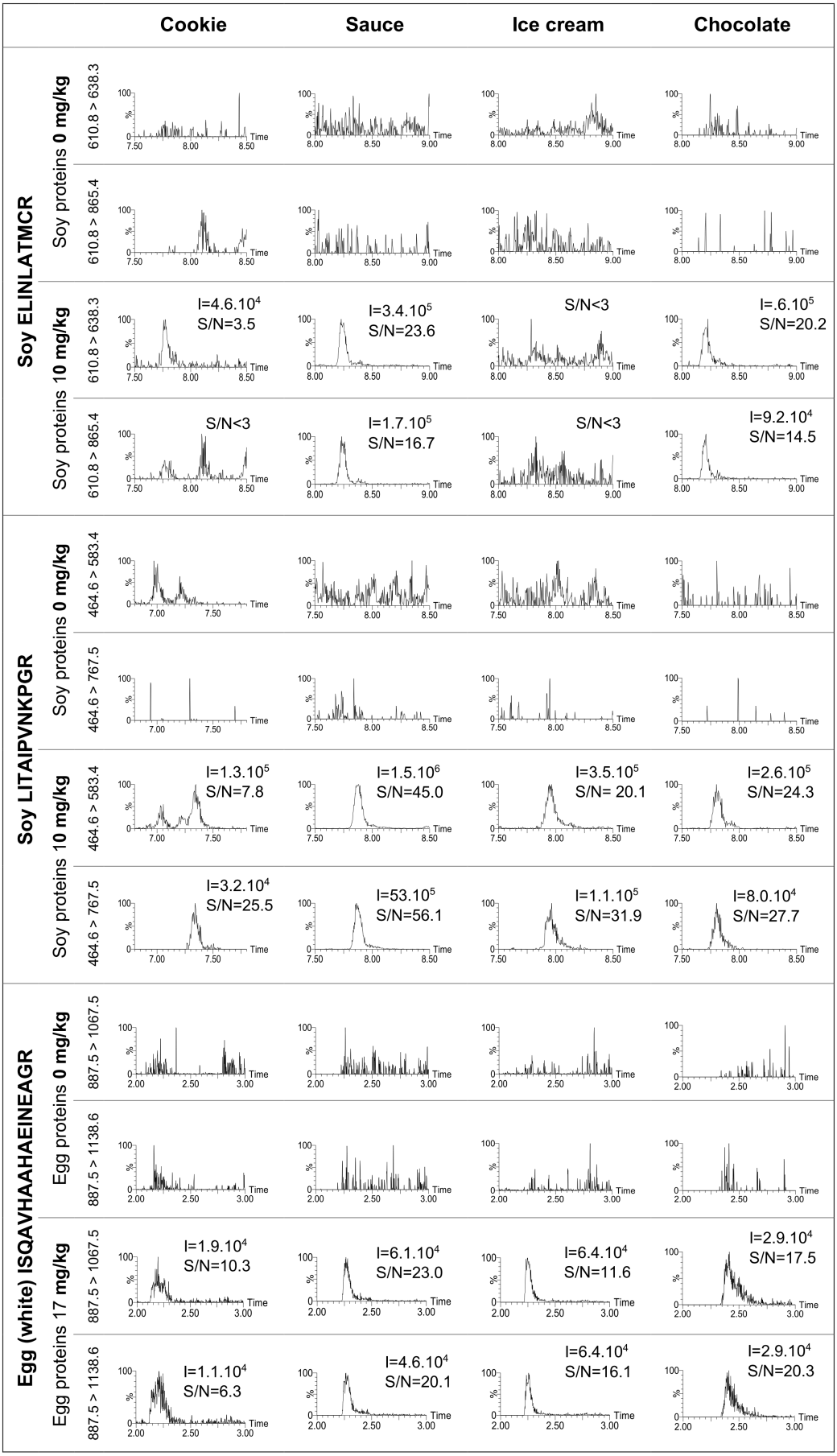
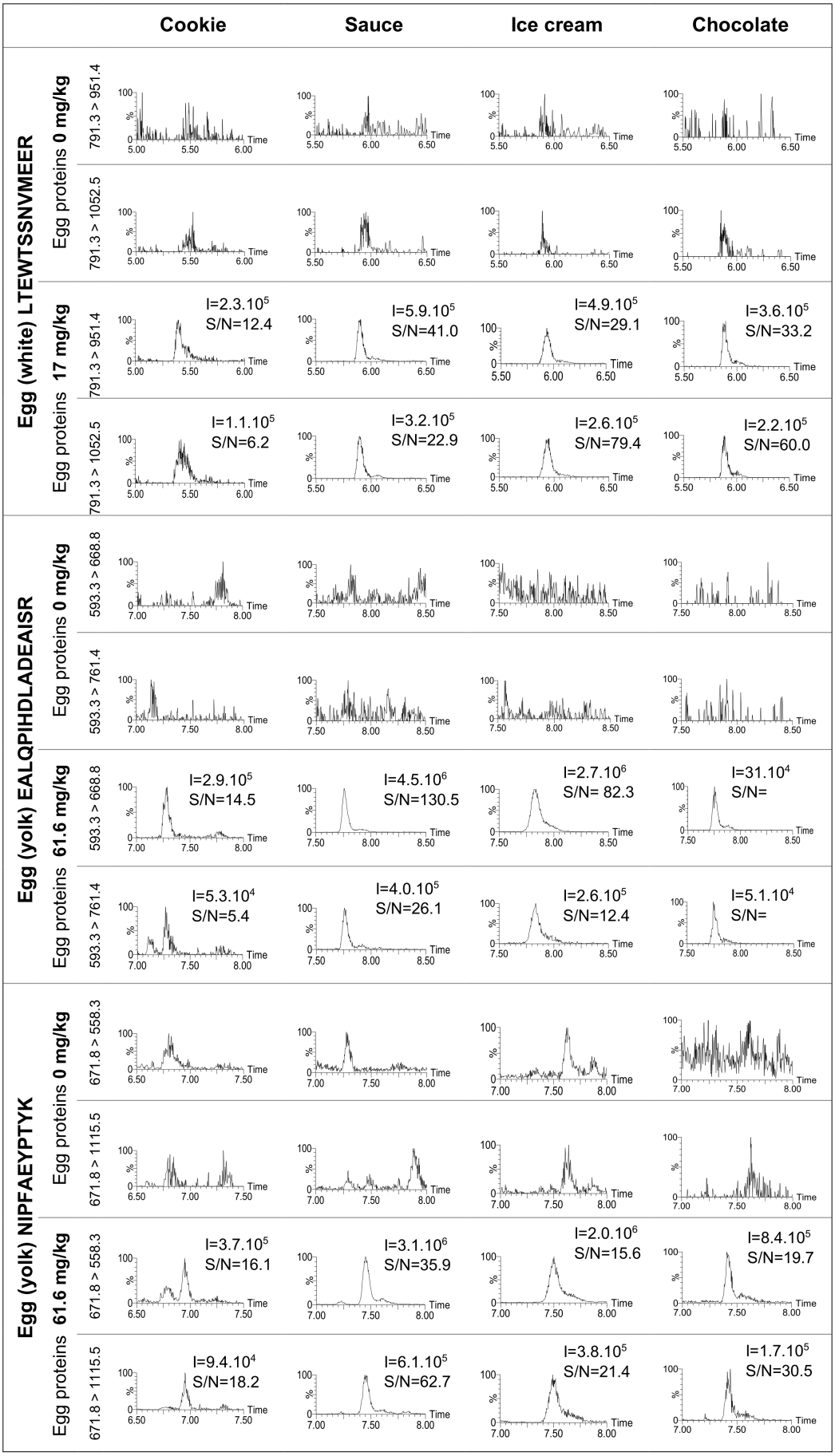
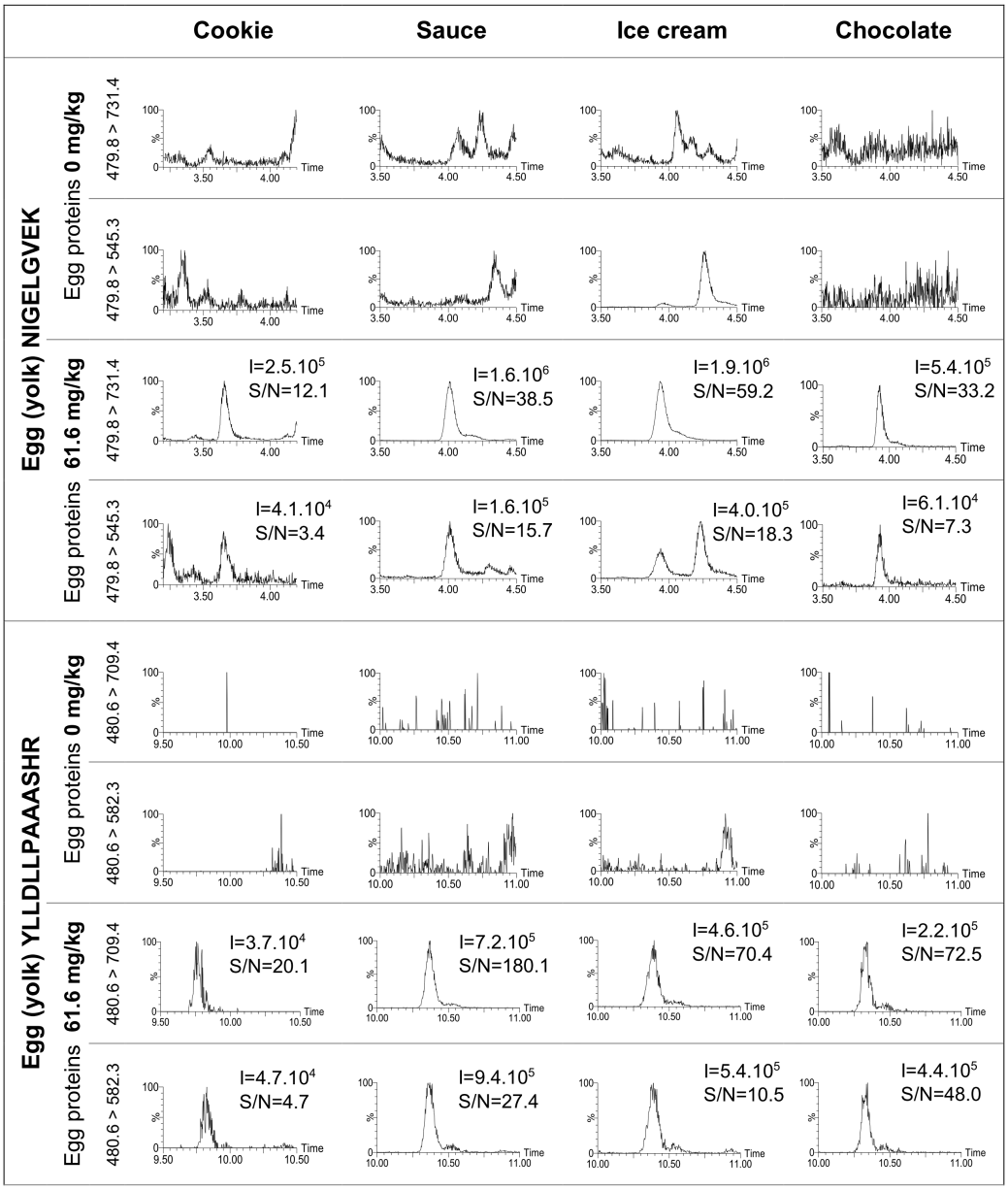
I=2.0.10^5 S/N=19.4


Cookie
Sauce
Ice cream
Chocolate
Soy ELINLATMCR
Soy proteins 0 mg/kg
610.8 > 638.3
610.8 > 865.4
Soy proteins 10 mg/kg
610.8 > 638.3
I=4.6.10^4
S/N=3.5
I=3.4.10^5
S/N=23.6
S/N<3
I=.6.10^5
S/N=20.2
610.8 > 865.4
S/N<3
I=1.7.10^5
S/N=16.7
S/N<3
I=9.2.10^4
S/N=14.5
Soy LITAIPVNKPGR
Soy proteins 0 mg/kg
464.6 > 583.4
464.6 > 767.5
Soy proteins 10 mg/kg
464.6 > 583.4
I=1.3.10^5
S/N=7.8
I=1.5.10^6
S/N=45.0
I=3.5.10^5
S/N= 20.1
I=2.6.10^5
S/N=24.3
464.6 > 767.5
I=3.2.10^4
S/N=25.5
I=53.10^5
S/N=56.1
I=1.1.10^5
S/N=31.9
I=8.0.10^4
S/N=27.7
Egg (white) ISQAVHAAHAEINEAGR
Egg proteins 0 mg/kg
887.5 > 1067.5
887.5 > 1138.6
Egg proteins 17 mg/kg
887.5 > 1067.5
I=1.9.10^4
S/N=10.3
I=6.1.10^4
S/N=23.0
I=6.4.10^4
S/N=11.6
I=2.9.10^4
S/N=17.5
887.5 > 1138.6
I=1.1.10^4
S/N=6.3
I=4.6.10^4
S/N=20.1
I=6.4.10^4
S/N=16.1
I=2.9.10^4
S/N=20.3


Cookie
Sauce
Ice cream
Chocolate
Egg (white) LTEWTSSNVMEER
Egg proteins 0 mg/kg
791.3 > 951.4
791.3 > 1052.5
Egg proteins 17 mg/kg
I=2.3.10^5 S/N=12.4
I=5.9.10^5 S/N=41.0
I=4.9.10^5 S/N=29.1
I=3.6.10^5 S/N=33.2
I=1.1.10^5 S/N=6.2
I=3.2.10^5 S/N=22.9
I=2.6.10^5 S/N=79.4
I=2.2.10^5 S/N=60.0
Egg (yolk) EALQPIHDLADEAISR
Egg proteins 0 mg/kg
593.3 > 668.8
593.3 > 761.4
Egg proteins 61.6 mg/kg
I=2.9.10^5 S/N=14.5
I=4.5.10^6 S/N=130.5
I=2.7.10^6 S/N= 82.3
I=31.10^4 S/N=
I=5.3.10^4 S/N=5.4
I=4.0.10^5 S/N=26.1
I=2.6.10^5 S/N=12.4
I=5.1.10^4 S/N=
Egg (yolk) NIPFAEYPTYK
Egg proteins 0 mg/kg
671.8 > 558.3
671.8 > 1115.5
Egg proteins 61.6 mg/kg
I=3.7.10^5 S/N=16.1
I=3.1.10^6 S/N=35.9
I=2.0.10^6 S/N=15.6
I=8.4.10^5 S/N=19.7
I=9.4.10^4 S/N=18.2
I=6.1.10^5 S/N=62.7
I=3.8.10^5 S/N=21.4
I=1.7.10^5 S/N=30.5


Cookie
Sauce
Ice cream
Chocolate
Egg (yolk) NIGELGVEK
Egg proteins 0 mg/kg
479.8 > 731.4
479.8 > 545.3
Egg proteins 61.6 mg/kg
I=2.5.10^5 S/N=12.1
I=1.6.10^6 S/N=38.5
I=1.9.10^6 S/N=59.2
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I=4.1.10^4 S/N=3.4
I=1.6.10^5 S/N=15.7
I=4.0.10^5 S/N=18.3
I=6.1.10^4 S/N=7.3
Egg (yolk) YLLDLLPAAASHR
Egg proteins 0 mg/kg
480.6 > 709.4
480.6 > 582.3
Egg proteins 61.6 mg/kg
I=3.7.10^4 S/N=20.1
I=7.2.10^5 S/N=180.1
I=4.6.10^5 S/N=70.4
I=2.2.10^5 S/N=72.5
I=4.7.10^4 S/N=4.7
I=9.4.10^5 S/N=27.4
I=5.4.10^5 S/N=10.5
I=4.4.10^5 S/N=48.0
Time
%

CHAPTER II

HIGHLIGHT ON BOTTLENECKS IN FOOD ALLERGEN ANALYSIS: DETECTION AND QUANTIFICATION BY MASS SPECTROMETRY

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Context

The substantial expansion of the allergic population has generated a high number of analytical methods, including ones based on mass spectrometry. Unfortunately, the lack of guidelines and regulations has led to significant divergences between methods used by different laboratories.

This publication was written to enable food laboratories to build awareness and understanding of the main bottlenecks in food allergen control and of their consequences for laboratories and industries.

The first bottleneck, shared by food industries and laboratories, is the lack of regulatory thresholds despite the quantity of data generated by clinical trials. The second, shared by clinicians and laboratories, is the lack of certified reference materials. This leads to the use of inappropriate and different standard materials. The last problem, specific to laboratories, is the use of methods for the analysis of processed samples without systematically conducting robustness studies to ensure that the method is valid for the detection of both raw and processed proteins in food products.

The first part of this paper provides examples of how the different bottlenecks affect the sensitivity of a developed detection method. It is of utmost importance to have harmonized regulations and methods for the detection of proteins in food allergens. In 2016, the first guideline for the detection of proteins of allergenic foods by mass spectrometry were published by an AOAC expert group, entitled “Standard Method Performance Requirements SMPR 2016.002” (Paez et al., 2016). This guideline, however, is incomplete, and some implementations are proposed in this article. The second part of the publication is dedicated to food protein quantification with the help of labeled peptides and to possible benefits of this approach for routine laboratories. The method described makes it possible to reduce the digestion time from 16 to 1 h and thus to analyze proteins in four allergens within a day. Its optimization is presented below, before the publication.

With a view to improving current regulations, the remaining gaps/problems are also discussed in two science popularization newsletter articles (AOAC International Newsletter and the Lab Info Newsletter n°16 of the Belgian Federal Agency for the Safety of the Food Chain (FASFC)) to sensitize food authorities and the general public. These newsletters are presented in Annexes 1 and 2 of the present thesis.

Preliminary optimization of the method

I Optimization of the tryptic digestion time

Optimization of the digestion time was performed by analyzing three independent preparations of incurred cookies (180 °C – 18 min) containing 20 mg milk proteins, 120 mg egg proteins, 200 mg soy proteins, and 100 mg peanut proteins per kg. Five different tryptic digestion times were tested: 1, 2, 3, 6, and 16 h.

I.I Sample preparation protocol

In each replicate, the proteins contained in 10 g cookie were extracted with 100 mL extraction buffer, (200 mM TRIS-HCl; pH 9.2, 2 M urea) by shaking at 20 °C for 30 min (Agitelec, France) followed by sonication for 15 min at 4 °C.

Extracts (50 mL) were diluted with 200 mM NH_4HCO_3 (50 mL) before reduction (5 mL - 800 mM DTT – 45 min) and protein alkylation (500 mM IAA – 45 min in the dark). The protein extracts were then split into 5 20-ml fractions in polypropylene tubes to assess the influence of the tryptic digestion time on peptide peak areas for milk, egg, soy, and peanut allergens. After tryptic digestion (1 mL trypsin at 1 mg/mL in 50 mM acetic acid) for 1 h to 16 h, the reaction was stopped by addition of 300 µL of 20% formic acid and the mixtures were kept at -20 °C prior to SPE clean-up performed the next day.

The peptide extract purification protocol described in the publication was followed (Planque et al., 2017 b) and the purified extracts analyzed by UHPLC-MS/MS.

I.II Results and discussion

The extraction and the beginning of the digestion were common to all conditions tested (1 to 16 h of digestion) in order to root out variability. **Figure 43** represents the peak areas for milk, egg, soy, and peanut peptides as a function of the tryptic digestion time.

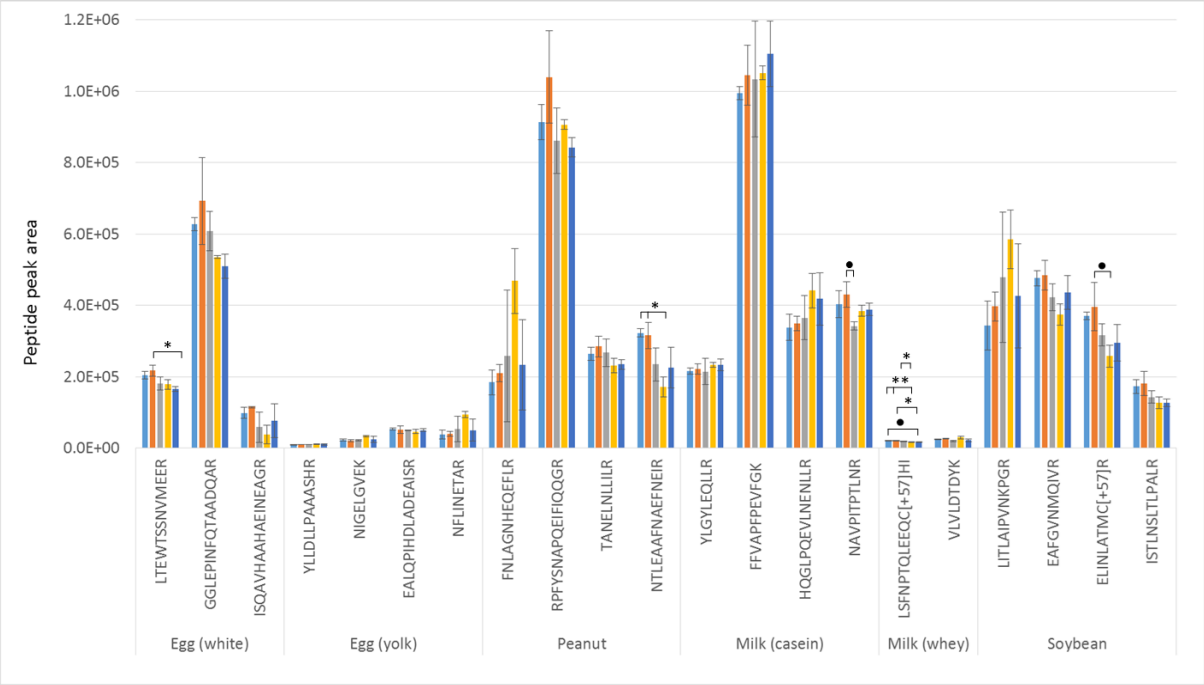

Figure 43: *Effects of the tryptic digestion time (1 h (light blue), 2h (orange), 3h (grey), 6 h (yellow) and 16h (dark blue) on the detected peptide peak areas for egg, peanut, milk, and soybean peptides. Results are expressed as mean areas ± 1 S.D. (n=3). Statistical analysis was performed by Student's t-test; p value: < 0.01 (**) < 0.05 (*) < 0.1 (●).*

Statistical analysis revealed the lack of any significant effect of the digestion time for 16 of the 21 peptides detected. The five peptides significantly impacted by the digestion time were: LTEWTSSNVMEER (egg), NTLEAAFNAEFNEIR (peanut), NAVPITPTLNR and LSFNPTQLEEQCHI (milk), and ELINLATMCR (soy). The peak areas corresponding to these five peptides were found to decrease as the digestion time increased. As no significant difference was observed between 1 and 2 h of trypsin digestion, both times can be chosen.

I.III Conclusion

The UHPLC-MS/MS method should be used in routine laboratories, and the samples should ideally be analyzed within a day. A 1-h digestion can advantageously replace the overnight digestion (16 h) used in the first publication presented in Chapter I.

EXTENDED ABSTRACTS

Highlight on Bottlenecks in Food Allergen Analysis: Detection and Quantification by Mass Spectrometry

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Food laboratories have developed methods for testing allergens in foods. The efficiency of qualitative and quantitative methods is of prime importance in protecting allergic populations. Unfortunately, food laboratories encounter barriers to developing efficient methods. Bottlenecks include the lack of regulatory thresholds, delays in the emergence of reference materials and guidelines, and the need to detect processed allergens. In this study, ultra-HPLC coupled to tandem MS was used to illustrate difficulties encountered in determining method performances. We measured the major influences of both processing and matrix effects on the detection of egg, milk, soy, and peanut allergens in foodstuffs. The main goals of this work were to identify difficulties that food laboratories still encounter in detecting and quantifying allergens and to sensitize researchers to them.

Based on a poster presented at the 130th AOAC Annual Meeting, September 18-21, 2016, in Dallas, TX

This project was financially supported by Waters Corp., the Walloon Region (FirstDoCA project: Allermass convention 1217881), and the Technological Platform Mass Spectrometry Service (MaSUN, UNamur, Belgium).

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Food allergies are increasingly prevalent, affecting over 220 million people worldwide (1). To avoid allergy, allergic consumers must exclude the prohibited food from their diet. Yet, despite many efforts and actions of the food industry, it is very hard to achieve complete elimination of cross-contact with allergens during food manufacturing, transport, and storage (2, 3). To limit the risk of allergy, the industry widely uses precautionary labeling (i.e., "may contain…"), but food recalls due to unlabeled allergens are constantly increasing (4). The lack of correlation between precautionary labeling and the presence of allergens frequently leads allergenic people to ignore the labeling (5). In addition, the absence of regulatory thresholds for allergens does not help food producers establish trustworthy labeling. To help food producers, thresholds have been set by the Allergen Bureau's Voluntary Incidental Trace Allergen Labeling (VITAL) Program (6, 7), but despite manufacturers' improvements and the emergence of allergen thresholds, it remains necessary to check for possible contaminations using reliable analytical methods.

Mélanie Planque was one of the five contest winners for first time AOAC poster presenters at the 130th AOAC Annual Meeting.

The ELISA is the test most widely used in routine laboratories to detect allergens in food products. Yet, detecting highly processed allergens at VITAL thresholds by ELISA is very difficult because of protein modifications and interfering compounds (polyphenols, high fat content, etc.; 8, 9). This limitation has led to the development of methods based on ultra-HPLC (UHPLC) coupled to tandem MS (MS/MS) for the detection of allergens in products processed at high temperature (10–12). Although several methods are available for detecting

allergens, a lack of harmonization between laboratories remains common. Although AOAC INTERNATIONAL *Standard Method Performance Requirements* (SMPR® 2016.002; 13) recommend using appropriate reference materials from the National Institute of Standards and Technology (NIST; Gaithersburg, MD) or from LGC Standards, some reference materials are still unavailable (cashew, pistachio, Brazil nut, and others), or food laboratories do not use them routinely. This lack of harmonization makes it difficult to compare method performances between laboratories. Food allergen detection methods are mostly characterized by sensitivity thresholds in spiked materials (14, 15), and it is hard to relate method sensitivity determined in this manner to method performances in food industry processes. Problems such as the absence of conversion factors between reporting units and the lack of correspondence between analytical methods such as PCR (DNA copies; 16) and ELISAs or MS (ingredients, soluble proteins, and total proteins; 10, 11) further complicate comparing the performances of methods used for food allergen detection. In the present study, we have sought to illustrate the consequences of this lack of harmonization between laboratories using UHPLC-MS/MS analysis. This technique was used to analyze highly processed and spiked materials. The impacts of processing and matrix effects were systematically evaluated and compared for the detection of egg, milk, soy, and peanut allergens in ice cream (fat), cookie (processed), and tomato sauce (acid) matrixes.

Materials and Methods

Reagents and Materials

Standard Reference Materials (SRMs) of whole egg powder (SRM 8445; 48% proteins), whole milk powder (SRM 1549a; 25.64% proteins), soy flour (SRM 3234; 53.37% proteins), and peanut butter (SRM 2387; 22.2% proteins) were from NIST. Ultra-performance LC (UPLC) grade acetonitrile and formic acid were from Biosolve Ltd (Valkenswaard, the Netherlands). Acetic acid was purchased from Acros Organics (Geel, Belgium) and hydrochloric acid from Fisher Chemical (Loughborough, United Kingdom). The cleanup step was performed with a Sep-Pak tC18 SPE column (Cat. No. WAT036790; 6 cc, 500 mg), and UPLC separation was done with a Peptide C18 BEH 130Å column (2.1 × 150 mm), both from Waters Corp. (Milford, MA). Urea, ammonium bicarbonate, tris(hydroxymethyl) aminomethane (TRIS), dimethyl sulfoxide (DMSO), dithiothreitol (DTT), iodoacetamide (IAA), bicinchoninic acid (BCA) protein assay kit, and trypsin from bovine pancreas (Cat. No. T8802) were obtained from Sigma-Aldrich (Bornem, Belgium). The labeled peptides GGLEPINF (Ring-D5) QTAADQAR-NH2 (egg peptide), FFVAPFPEVFGK (U-13C6 15N2)-OH (milk peptide), EAFGV (D8)NMQIVR-OH (soy peptide), and TANELNLLIL (U-13C6 15N)*R*-OH (peanut peptide), as internal standards, were from Eurogentec (Seraing, Belgium).

Preparation of Food Samples and Standards

Allergen-free matrixes.—Cookie dough was prepared with flour (53.4%), sugar (15.2%), water (14.8%), oil (16.1%), salt (0.3%), ammonium bisulfate (0.1%), and sodium bicarbonate (0.1%). Ice cream was mixed in a blender and consisted of coconut milk (29.6%), sugar (11.0%), lemon juice (0.4%), and banana (59.0%). Tomato sauce [tomatoes (75%), onions, carrots, and celery] was purchased from a local shop and homogenized with a blender.

Allergen solutions.—Two allergen solutions (Solutions A and B) were prepared in extraction buffer (200 mM TRIS-HCl; pH 9.2, 2 M urea) and used to spike matrixes at different concentrations. Solution A was prepared by combining all four NIST reference materials (egg powder, milk powder, soy flour, and peanut butter) with the extraction buffer, applying the extraction protocol to this mixture, centrifuging it, and retaining the final supernatant. Ingredient and buffer proportions were chosen so that 1 mL Solution A corresponded, on the basis of NIST protein content, to 0.6 mg total egg proteins, 0.1 mg total milk proteins, 1.0 mg total soy proteins, and 0.5 mg total peanut proteins. Solution B was prepared by extracting each starting material separately, measuring the soluble protein content of each extract (BCA determination), and combining the different extracts so that 1 mL Solution B contained 0.1 mg soluble milk proteins, 0.6 mg soluble egg proteins, 1.0 mg soluble soy proteins, and 0.5 mg soluble peanut proteins. The allergen concentrations of these two solutions were, therefore, not identical, although both can be described as containing, e.g., "milk proteins at 0.1 mg/mL." In what follows, we therefore specify "total proteins" or "soluble proteins" when referring to spiking levels obtained with Solution A or Solution B, respectively.

Contaminated matrixes.—Three kinds of contaminated matrixes were prepared: incurred (incorporation of allergens before processing at 180°C during 18 or 36 min); spiked (incorporation of allergens after processing of tomato sauce, ice cream, and cookie matrixes); and digested (spiking the tomato sauce, ice cream, and cookie matrixes with digested allergens after the digestion step). Calibration curves (n = 3) were constructed for determining protein concentrations in milligrams of total proteins per kilogram (NIST protein content) or in milligrams of soluble proteins per kilogram (BCA quantification). The concentrations used were 0, 3, 6, 15, 30, 60, and 120 mg/kg for egg; 0, 0.5, 1, 2.5, 5, 10, and 20 mg/kg for milk; 0, 5, 10, 25, 50, 100, and 200 mg/kg for soy; and 0, 2.5, 5, 12.5, 25, 50, and 100 mg/kg for peanut.

Internal standards.—The stock solution was prepared by dissolving the labeled peptides in DMSO (10 mg/mL) before diluting to 1 mg/mL with 0.1% formic acid. The working solution, containing FFVAPFPEVFGK (U-13C6 15N2)-OH and EAFGV (D8)NMQIVR-OH at 4 µg/mL, TANELNLLIL (U-13C6 15N)*R*-OH at 10 µg/mL, and GGLEPINF (Ring-D5) QTAADQAR-NH2 at 8 µg/mL in 0.1% formic acid, was prepared using the stock solution.

Peptide Analysis Protocol

Incurred and spiked matrixes.—For extraction, digestion, purification, and analysis by UHPLC-MS/MS, the protocol described by Planque et al. (12) was used. Before extraction, 75 µL labeled-peptide working solution was added to each spiked and incurred matrix sample. The digestion step was slightly modified: 10 mL supernatant was diluted in 10 mL 0.20 mol/L

ammonium bicarbonate. The proteins were reduced for 45 min at 20°C by adding 1 mL 0.80 mol/L DTT, alkylated for 45 min at 20°C in the dark by adding 2 mL 0.50 mol/L IAA, and digested at 37°C for 1 h by addition of 1 mL 1 mg/mL trypsin in 50 mM acetic acid. The reaction was stopped with 300 µL 20% formic acid, and the mixture centrifuged at 4660 × *g* for 5 min at 10°C. After purification, the extract was dissolved in 500 µL 0.1% formic acid and centrifuged (11 754 × *g* for 5 min) before analysis by UHPLC-MS/MS.

Digested matrixes.—Tomato sauce, ice cream, and cookie matrixes were spiked with Solution A (described in the *Allergen solutions* section) and digested according to the above-described protocol. After spiking the digested matrixes with digested allergens, 37.5 µL labeled-peptide working solution was added. After purification, the extract was dissolved in 500 µL 0.1% formic acid and analyzed by UHPLC-MS/MS.

Results and Discussion

Validation Guidelines: Impact on Method Performance

Despite the establishment of guidelines, validation criteria are still fuzzy. In particular, laboratories can still choose the manner in which they determine method sensitivity. We examined how different choices might affect the determined sensitivity of UHPLC-MS/MS.

First, we focused on egg proteins in the incurred and the spiked cookie matrix. In UHPLC-MS/MS, the sensitivity (LOQ) is defined as the lowest concentration of analyte corresponding to an S/N higher than 10. In the incurred cookie matrix, having undergone heating at 180°C for 18 min, the LOQ was approximately 3 mg total egg proteins/kg (Figure 1B). When processing was longer (36 min at 180°C), the threshold S/N value was not reached at this concentration (Figure 1A). The use of spiked rather than incurred cookie matrix increased the S/N ratio nearly 7-fold (Figure 1C versus Figure 1A), and when the 3 mg/kg value referred to milligrams of soluble proteins rather than total proteins, the S/N ratio was even higher (Figure 1D).

These results highlight the difficulty in comparing method sensitivities when the reporting units are not the same and when different choices are made regarding processing conditions and the use of spiked or incurred samples. Such discrepancies are an obstacle to guaranteeing reliable detection of allergens in foods.

Next, we looked at the three matrixes (tomato sauce, ice cream, and cookie) spiked with 0.5 mg/kg total milk proteins. The sensitivity of the method was found to depend greatly on the matrix used: the SN was 3.2 times as high in ice cream and 8.8 times as high in tomato sauce as in cookie (Figure 2).

These results show that the matrix effect can be considerable and that foodstuff composition must be taken into account when assessing method performance. AOAC SMPR 2016.002 recommends combining matrixes and allergens when validating a method. It does not, however, indicate which ingredients, proportions, and conditions of preparation should be used. For harmonization, reference materials (e.g., MoniQA, LGC Standards) should be tested/used by different laboratories in order to use the same materials in determining the performances of analytical methods for food allergens.

Quantification Strategies for Allergens: Comparing Different Approaches

For better management of cross-contaminations in production lines, the food industry requires quantitative data, but industrial processes are known to cause protein modifications and degradations, making it hard to deliver accurate quantitative results (17). In the ELISA technique, the quantification of allergens is based mainly on a single calibration curve done in a solvent or extracted matrix, no matter what kind of food matrix is to be analyzed (18). This means that matrix effects and thermal processes are usually not taken into account and leads to significant gaps between real and quantified amounts of allergens.

These considerations led us to examine the utility of using labeled peptides as internal standards in spiked and incurred materials to be analyzed by MS (Figure 3). First, such standards were introduced before extraction so as to test their ability to correct for matrix-related effects and steps of the protocol.

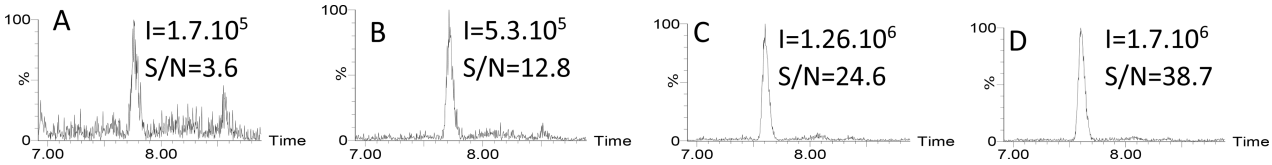

Figure 1. Chromatograms obtained for the most abundant multiple reaction monitoring (MRM) transition for egg peptide GGLEPINFQTAADQAR (844.4 > 666.3) in cookie matrix: (A-B) incurred samples containing 3 mg total egg proteins/kg cookie (NIST content) processed at 180°C for (A) 18 min or (B) 36 min; (C-D) spiked samples at a spiking level of 3 mg egg proteins/kg; however, this value refers to total proteins (NIST content) in panel C and to soluble proteins (BCA determination) in panel D.

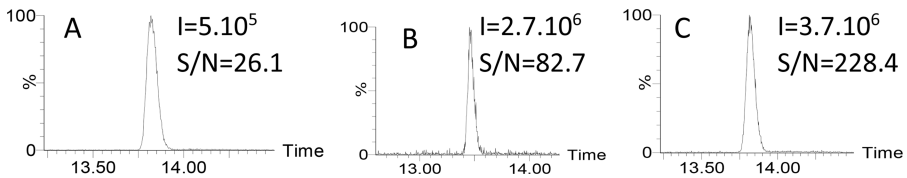

Figure 2. Chromatograms obtained for the most abundant MRM transition for milk peptide FFVAPFPEVFGK (692.9 > 920.5) in spiked matrixes of (A) cookie, (B) ice cream, and (C) tomato sauce. The spiking level was 0.5 mg total milk proteins/kg food product (i.e., the LOQ determined in cookie matrix after processing at 180°C for 18 min; 12).

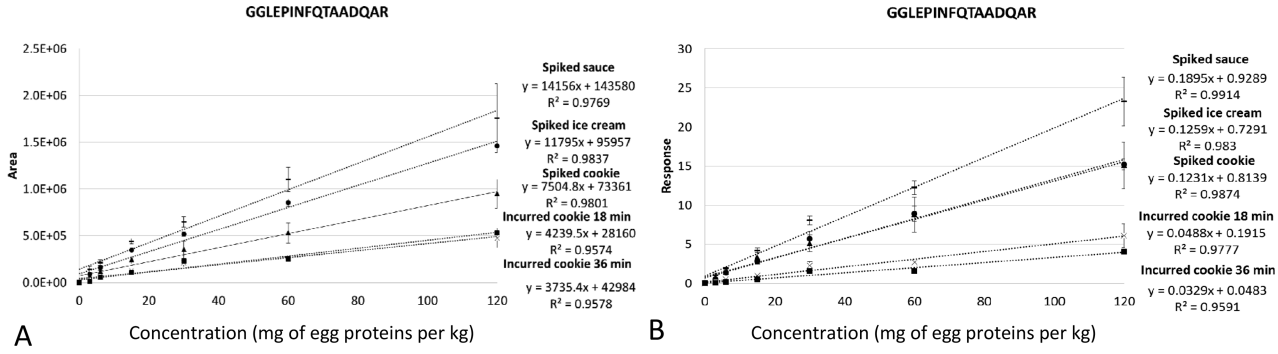

Figure 3. Linear regression of peptide peak areas and responses (most abundant MRM transition) for egg peptide GGLEPINFQTAADQAR (844.4 > 666.3) as a function of the concentration of egg proteins per kilogram of spiked: tomato sauce, ice cream, and cookie matrixes, as well as incurred cookie matrix processed at 180°C for 18 and 36 min (A) without internal standard and (B) with internal standard correction GGLEPINF (Ring-D5)QTAADQAR-NH2 (response = area of peptide ÷ area of internal standard).

As expected, the use of a labeled peptide did not allow correcting for the effect of heating. Nor did it allow the use of a single curve for ice cream, cookie, and tomato sauce matrixes. To see whether the use of labeled peptides as internal standards would allow correcting for matrix-related effects occurring during purification/analysis, matrixes were extracted and digested before being spiked with digested allergens and labeled peptides. As shown in Figure 4, the calibration curves for cookie, tomato sauce, and ice cream matrixes were found to coincide. These results

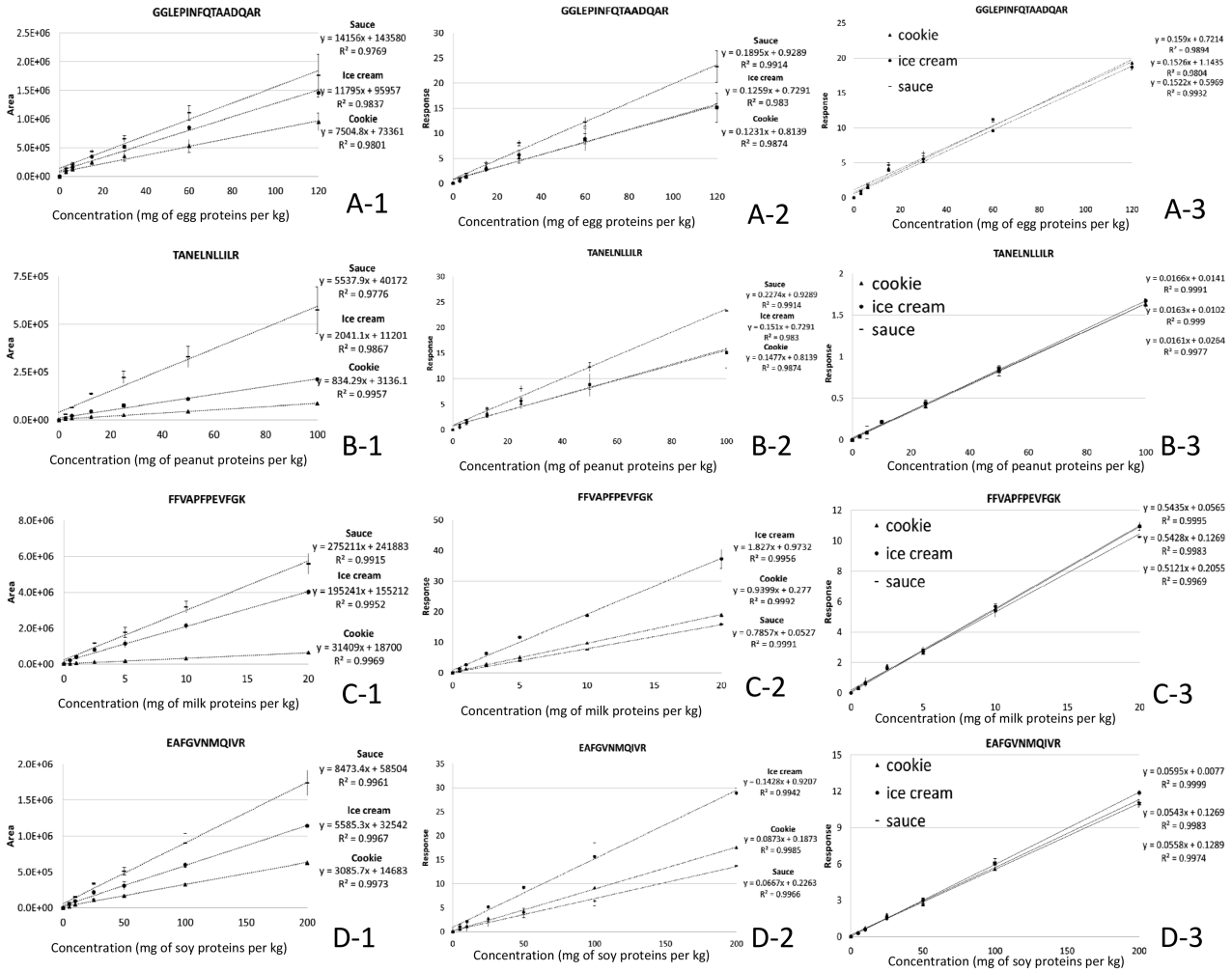

Figure 4. Linear regression of the most abundant MRM transitions for (A) egg peptide GGLEPINFQTAADQAR (844.4 > 666.3), (B) peanut peptide TANELNLLILR (635.4 > 741.5), (C) milk peptide FFVAPFPEVFGK (692.9 > 920.5), and (D) soy peptide EAFGVNMQIVR (632.3 > 760.4), based on (A1–D1) peak areas, (A2–D2) responses with the labeled peptide correction (response = area of peptide ÷ area of labeled peptide internal standard), and (A3–D3) responses with the labeled peptide correction for digested matrixes spiked with digested peptides.

are encouraging, but additional strategies are required for correcting extraction and digestion steps.

These results support the use of labeled peptides as internal standards, but correcting for effects occurring during extraction/digestion seems crucial to allowing the use of a single calibration curve to quantify an allergen in various foodstuffs. Pending a solution for achieving this, alternatives must be found. Currently, the method of standard addition seems the most appropriate for quantifying allergens by UHPLC-MS/MS.

Conclusions

The aim of the present study was to list and measure gaps in food allergen detection and quantification, using UHPLC-MS/MS. We have demonstrated the importance of determining method performance in different matrixes under conditions similar to those of industrial manufacturing in order to guarantee the detection of allergens in real food products. For global harmonization, reference materials should be tested and used by the different food allergen control laboratories. We have further found that for main egg, milk, soy, and peanut peptides, the use of labeled peptides does not correct for matrix effects linked to the extraction and digestion steps, but it does perfectly correct for matrix effects, purification, and UHPLC-MS/MS analysis. Therefore, right now, it seems that adding an allergen standard for each foodstuff is the best way to quantify allergens. With the aim to develop a quantitative method with a single calibration curve, concatenated labeled peptides will also be tested in the near future.

Acknowledgments

We would like to thank the AOAC INTERNATIONAL Technical Programming Committee for the poster presentation price and the opportunity to publish an extended abstract. We also thank Waters Corp., the Walloon Region (FirstDoCA project: Allermass convention 1217881), and the Technological Platform Mass Spectrometry Service (University of Namur, Belgium) for scientific discussions regarding this project.

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CHAPTER III

LIQUID CHROMATOGRAPHY COUPLED TO TANDEM MASS SPECTROMETRY FOR DETECTING TEN ALLERGENS IN COMPLEX AND INCURRED FOODSTUFFS

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Context

Worldwide, tree nuts are responsible for severe anaphylaxis reactions. The method described in the second chapter has been implemented and extended to one of the most problematic families of food allergens causing life-threatening reactions.

The six most prevalently consumed tree nuts were added to the list of allergens analyzed: walnut, almond, cashew, pecan nut, pistachio, and hazelnut. We now show that the method we developed is able to detect, with a single protocol, peptides generated by the digestion of proteins of ten allergens. Data was obtained for two processed (cookie and tomato sauce) and two complex food products (chocolate and ice cream, the former containing tannins and the latter fat).

More than a year after our first publication presented in Chapter I, to the best of our knowledge, only one additional study has been published for the sensitive detection of peptides of several allergens in processed food products. The method, based on a UHPLC-MS/MS method of Pilolli et al. (2016) has LODs (S/N>3) expressed in mg of proteins per kg, of 2.5 for milk, 4.3 for egg, 3.2 for soy, 3.3 for peanut, and 1.1 for hazelnut in processed cookie (200 °C-12 min). The sensitivities of the methods described by both Pilolli et al. (2017) and Planque et al. (2016) support the adequacy of mass spectrometry for the detection of peptides of several allergens in processed food products (Planque et al., 2016; Pilolli et al., 2017).

In this publication, the digestion step was optimized to allow sample analysis within a day, in order to provide a routine method rivalling the performance of ELISAs. Furthermore, to allow the analysis of samples without major fouling of the ion source of the mass spectrometer, the extracts used were more diluted (in 600 µl instead of 200 µl acetonitrile (ACN)/0.1% formic acid (95:5 v/v)). Another measure taken to reduce fouling, as in UHPLC-MS/MS the flow can be directed to the waste or to the MS instrument for analysis, was to modify slightly the UHPLC gradient to increase the wasting time before injection into the mass spectrometer.

Major achievements

The optimization of the method significantly enhanced the efficiency of this routine method. The main added value of this work was to allow high-sensitivity detection of peptides obtained for 10 allergens within a day, in several food products including processed ones. Using a single protocol and considering a signal-to-noise ratio higher than 10 and 3 for the first and second most abundant multiple reaction monitoring (MRM) transition, respectively, we were able to detect target allergens at 0.5 mg/kg for milk proteins, 2.5 mg/kg for peanut, hazelnut, pistachio, and cashew proteins, 3 mg/kg for egg proteins, and 5 mg/kg for soy, almond, walnut, and pecan proteins. The method was

also used to analyze allergens which the ELISA approach had failed to detect, mainly because of major matrix interferences. For these reasons, the developed method is an efficient complementary approach for allergen detection, which can rapidly be implemented in routine laboratories.

Preliminary optimization of the method

I Optimization of the digestion step

1,4-Dithiothreitol (DTT) is toxic, so its concentration should be kept as low as possible for daily use in a routine laboratory. An alternative is to use tris (2-carboxyethyl)phosphine (TCEP), which is less toxic than DTT but must still be used with caution because of its corrosive properties.

Protein digestion was optimized by analyzing peptides in three independent preparations of incurred cookie (180 °C – 18 min) containing 20 mg milk proteins, 120 mg egg proteins, 200 mg soy proteins, and 100 mg peanut proteins per kg.

I.I Optimization of the DTT concentration

To avoid variability and to allow comparisons on the same material, the amounts of sample material were adapted so as to use the same protein extracts for the different digestion conditions.

After protein extraction (200 mM Tris-HCl pH 9.2, 2 M urea), the proteins contained in 10 mL supernatant were diluted twice with 10 mL digestion buffer (200 mM ammonium bicarbonate NH_4HCO_3). DTT at concentrations ranging from 10 mM to 80 mM were tested for the reduction of disulfide bonds of allergenic proteins contained in incurred cookies (incubation for 45 min at 20 °C). Protein alkylation with iodoacetamide (IAA) was performed for 45 min at 20 °C in the dark, and IAA (at concentrations ranging from 20 mM to 160 mM) was used to block free cysteines. Enzymatic digestion was performed by adding 1 mL trypsin (1 mg/mL in 50 mM acetic acid) and the reaction mixture was incubated at 37 °C for 1 h. The digestion reaction was stopped by addition of 300 µL of 20% formic acid.

Peptides of milk, egg, soy, and peanut allergens were analyzed by UHPLC-MS/MS (Xevo TQS – Waters) to determine the effect of the DDT concentration on the peak areas corresponding to target peptides (**Figure 44**).

As the DTT concentration increased, the peak areas corresponding to the four peptides YLLDLLPAAASHR, NIGELGVEK and NFLINETAR (egg yolk), and HQGLPQEVLNENLLR (milk casein) were significantly decreased. Those corresponding to the three peptides ELINLATMCR (soy), LSFNPTQLEEQCHI (milk whey), and NTLEAAFNAEFNEIR (peanut) were significantly increased.

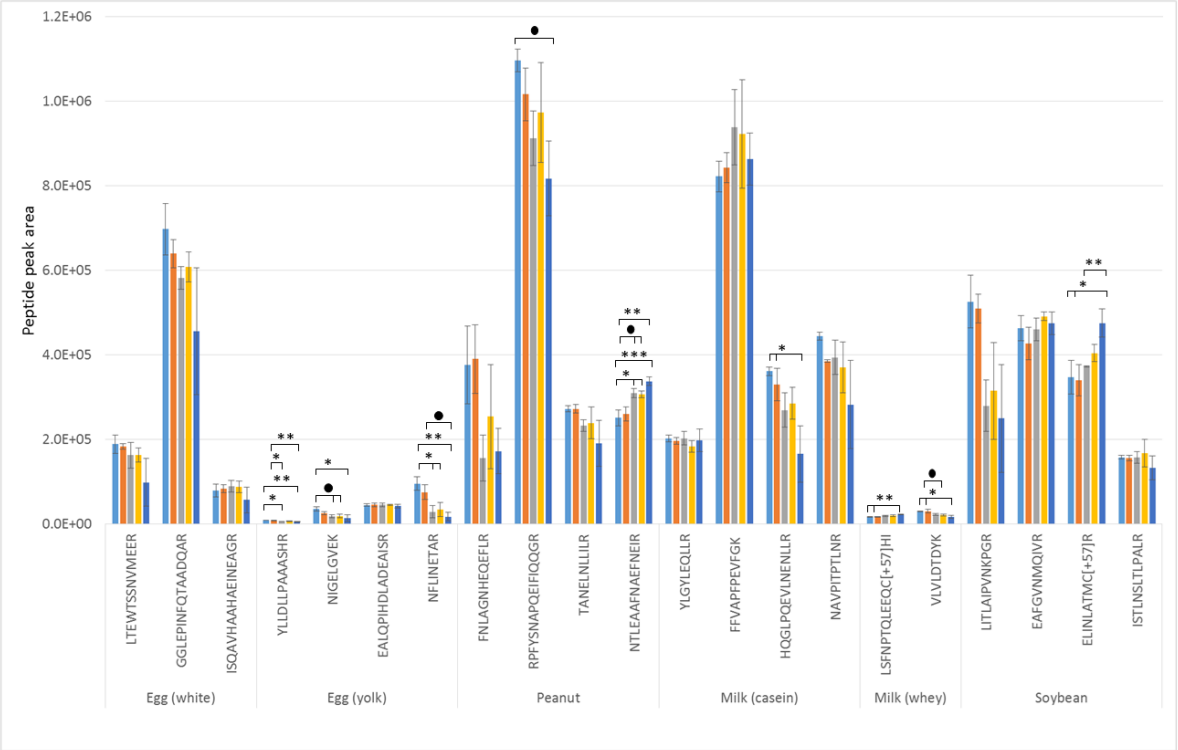

***Figure 44**: Effect of the DTT concentration on the peak areas corresponding to milk, egg, soy, and peanut peptides in incurred cookies (180 °C – 18 min) containing 20 mg milk proteins, 120 mg egg proteins, 200 mg soy proteins and 100 mg peanut proteins per kg. Reduction of protein disulfide bonds was performed with 10 (light blue), 20 (orange), 40 (gray), 60 (yellow), and 80 mM DTT (dark blue) followed by addition of 20, 40, 80, 120, and 160 mM IAA. Results are expressed as means ± 1 S.D and statistical analysis was performed with Student's test (n=3 independent extractions): p value: < 0.001 (***), < 0.01 (**), < 0.05 (*), < 0.1 (●).*

Because of the toxicity of DTT and the decrease of the peak areas of four peptides with a higher concentration of DTT, the selected concentrations were 10 mM DTT and 20 mM IAA.

I.II Comparison of TCEP and DTT as disulfide bond reducers

The reduction of disulfide bonds in proteins was compared between TCEP (Thermo Fisher ref: 20490) and DTT (Sigma Aldrich ref: D0632) (**Figure 45**). As the choice of a good reducing agent is application specific, the efficiencies of both had to be tested on the target proteins (Getz et al., 1999).

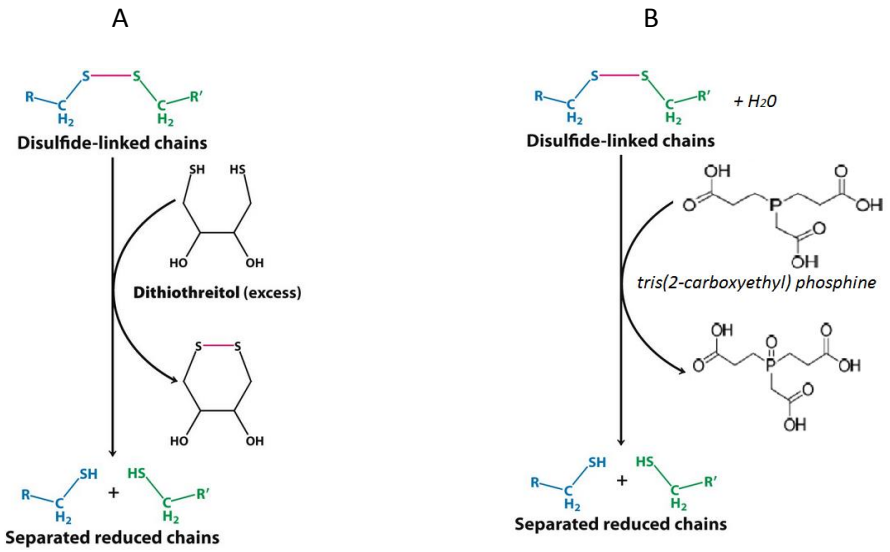

***Figure 45**: Mechanism of disulfide bond reduction by (A) DTT (1, 4-dithiothreitol) and (B) TCEP (tris(2-carboxyethyl)phosphine) (modified from* (Berg et al., 2012)*).*

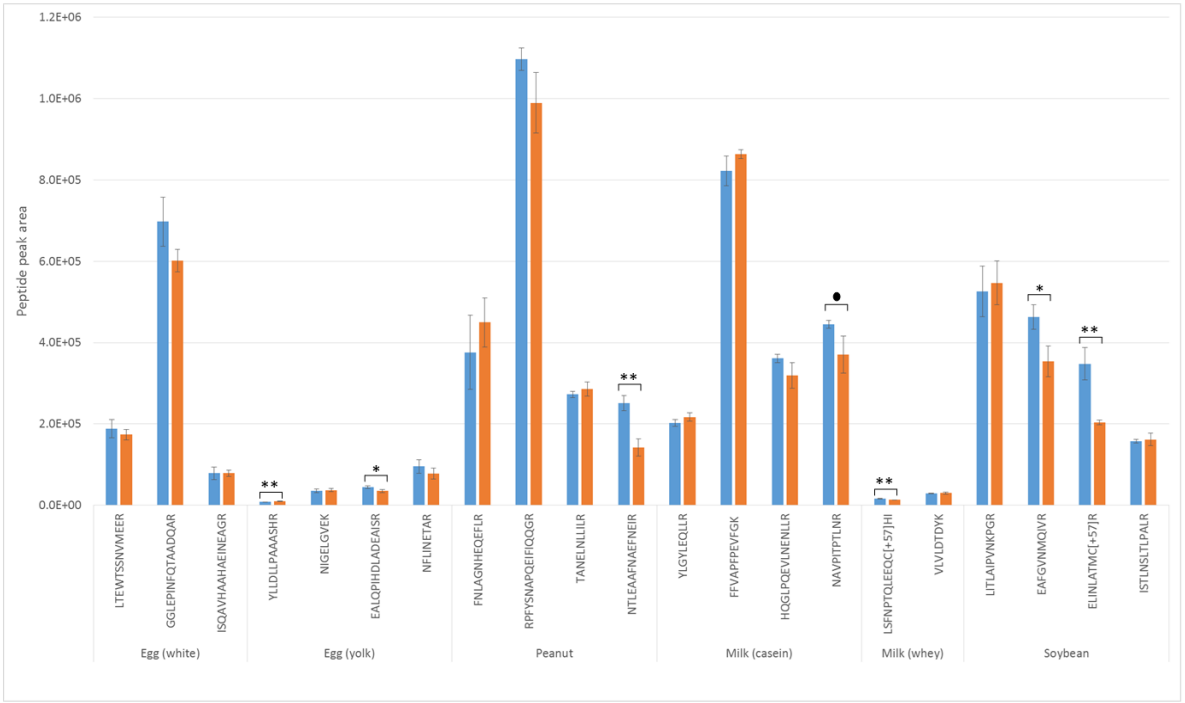

***Figure 46 :** Effect of the reducing agents DTT (10 mM, blue column) and TCEP (10 mM, orange column) on the peak areas for milk, egg, soy, and peanut peptides in incurred cookies (180 °C – 18 min) containing 20 mg milk proteins, 120 mg egg proteins, 200 mg soy proteins and 100 mg peanut proteins per kg. Proteins were incubated in the presence of 10 mM DTT or TCEP, followed by addition of 20 mM IAA and 50 µg trypsin. Results are expressed as means ± 1 S.D and statistical analysis was performed with Student's test (n=3 independent extractions): p value: < 0.01 (**), < 0.05 (*), < 0.1 (●).*

A comparison performed by UHPLC-MS/MS of TCEP and DTT used as reducing agents at 10 mM concentration (followed by treatment with 20 mM IAA) showed a significant improvement of the

peak areas for 6 of the 21 peptides when DTT was used as a reducing agent, while only one peptide peak area was found to be increased (YLLDLLPAAASHR) when TCEP was used (**Figure 46**).

It thus appeared that when used at 10 mM, DTT leads to larger peptide peak areas for egg, milk, peanut and soy peptides. This reducing agent was thus kept in the method for the reduction of disulfide bonds in proteins.

II Optimization of peptide extract purification

Under the experimental conditions used previously, major fouling of the source of the mass spectrometer was observed after running about 50 samples. To limit fouling problems, several parameters were optimized in order to obtain cleaner extracts, keeping in mind that the methods developed are for use in routine laboratories.

The extraction buffer and digestion conditions determined as described above were unchanged, but the amounts of the sample material were adjusted in order to use the same digested extracts for the different test conditions to avoid external variability.

The purification step is described in Chapter I: "*Digested proteins were purified on tC18 SPE columns. Cartridge pre-conditioning was performed with 18 ml ACN followed by equilibration with 18 ml of 0.1% formic acid. The digested proteins were centrifuged at 4660 g and 4 °C for 5 min and 20 ml supernatant was loaded on the column. Next, 18 ml of 0.1% formic acid was used to flush out impurities. Elution was then performed with 1.8 ml ACN/0.1% formic acid (30:70 v/v) followed by 3.6 ml ACN/0.1% formic acid (80:20 v/v). Before evaporation at 40 °C under a nitrogen flow, 30 µl DMSO (dimethyl sulfoxide) was added to avoid dryness. After evaporation, the pellets were resuspended in 200 µl of 0.1% formic acid and centrifuged for 5 min at 11754 g*".

Several conditions were tested with a view to improving sample purification:

- the **SPE cartridge**: (1) tC18 or (2) C18 to improve the sensitivity of the method
- the **washing solvent for SPE**: (1) 0.1% formic acid or (2) ACN/ 0.1% formic acid (5:95 v/v) to decrease fouling by samples
- the **evaporation temperature**: (1) 40 °C or (2) 60 °C to decrease the evaporation time
- the **peptide elution from the SPE column**: (1) 1.8 ml of ACN/0.1% formic acid (30:70 v/v) followed by 3.6 ml ACN/0.1% formic acid (80:20 v/v) or (2) 6 ml ACN/0.1% formic acid (80:20 v/v). The percentage of ACN was increased with a view to improving the elution of highly hydrophobic peptides and hence the peptide peak areas observed after UHPLC-MS/MS analysis

For each condition, the results were obtained from 3 independent cookie preparations. The extracts were diluted in 600 µl of ACN/0.1% formic acid (5:95 v/v) to decrease fouling and to be near the initial conditions of the UHPLC gradient. The clean-up protocol described in chapter I was followed and optimized. At each optimization step, the optimal conditions determined at the previous step were implemented.

II.I Selecting the clean-up cartridge

As described in Chapter I, two SPE cartridges, tC18 and HLB, were tested. The tC18 cartridge was chosen.

The results obtained with the C18 SPE column were compared with those obtained with the tC18 column. The particle size and sorbent are different between the two columns, but all other technical specifications are identical (**Table 6**).

	tC18 (**WAT036790)**	**C18 (WAT043395)**
Mode	Reversed phase – End Capped	Reversed phase – End Capped
Sorbent	Silica - $SiC_{18}H_{37}$	Silica - $Si(CH_3)_2C_{18}H_{37}$
Sorbent per cartridge	500 mg	500 mg
Particle size	37 – 55 µm	55 – 105 µm
pH range	2 - 8	2 - 8
Pore size	125 Å	125 Å

Table 6*: Technical specifications of the tC18 and the C18 solid phase extraction columns from Waters.*

After UHPLC-MS/MS, we found the peak areas for 7 of the 21 peptides (2 for egg, 1 for peanut and 4 for milk) to be significantly better with the C18 column than with the tC18 column (**Figure 47**). This difference could be explained by the grafting of silica sorbent.

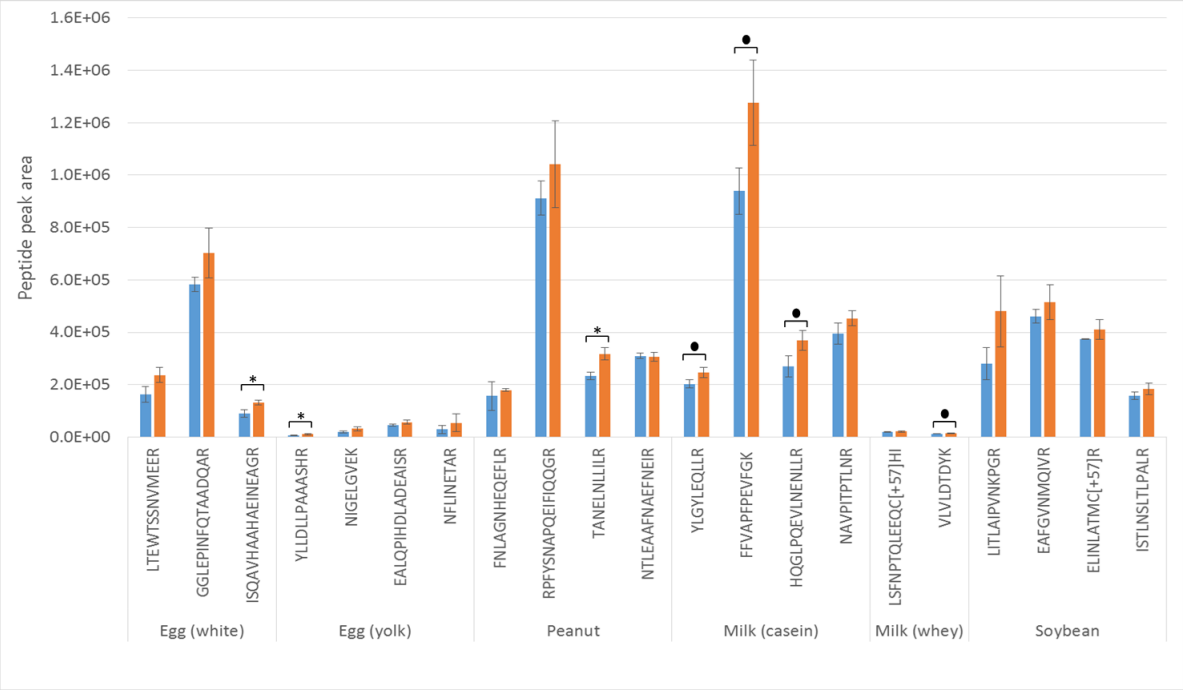

Figure 47: *Comparison of two solid phase extraction columns (tC18, blue column and C18, orange column). Effect of column choice on the mean peptide peak areas for milk, egg, soy, and peanut in incurred cookies (180 °C – 18 min) containing 20 mg milk proteins, 120 mg egg proteins, 200 mg soy proteins, and 100 mg peanut proteins per kg. Results are expressed as means ± 1 S.D and statistical analysis was performed with Student's test (n=3 independent extractions): p value: < 0.05 (*), < 0.1 (●).*

On the basis of the greater peak areas obtained for 7 peptides, the C18 cartridge was chosen for peptide purification.

II.II SPE cartridge washing

To flush out impurities, 0.1% formic acid was used. A small percentage of the organic solvent ACN was added (ACN/0.1% formic acid (5:95 v/v)) so as to promote elimination of weakly polar impurities.

The analysis of digested extracts, obtained with the different washing solvents, by mass spectrometry showed slightly but significantly higher peak areas for three egg peptides (LTEWTSSNVMEER, ISQAVHAAHAEINEAGR and NFLINETAR) and one milk peptide (VLVLDTDYK) when ACN/0.1% formic acid (5:95 v/v) was used (**Figure 48**). Under these conditions, however, one egg peptide (GGLEPINFQTAADQAR) and one soy peptide (ELINLATMCR) showed reduced peak areas.

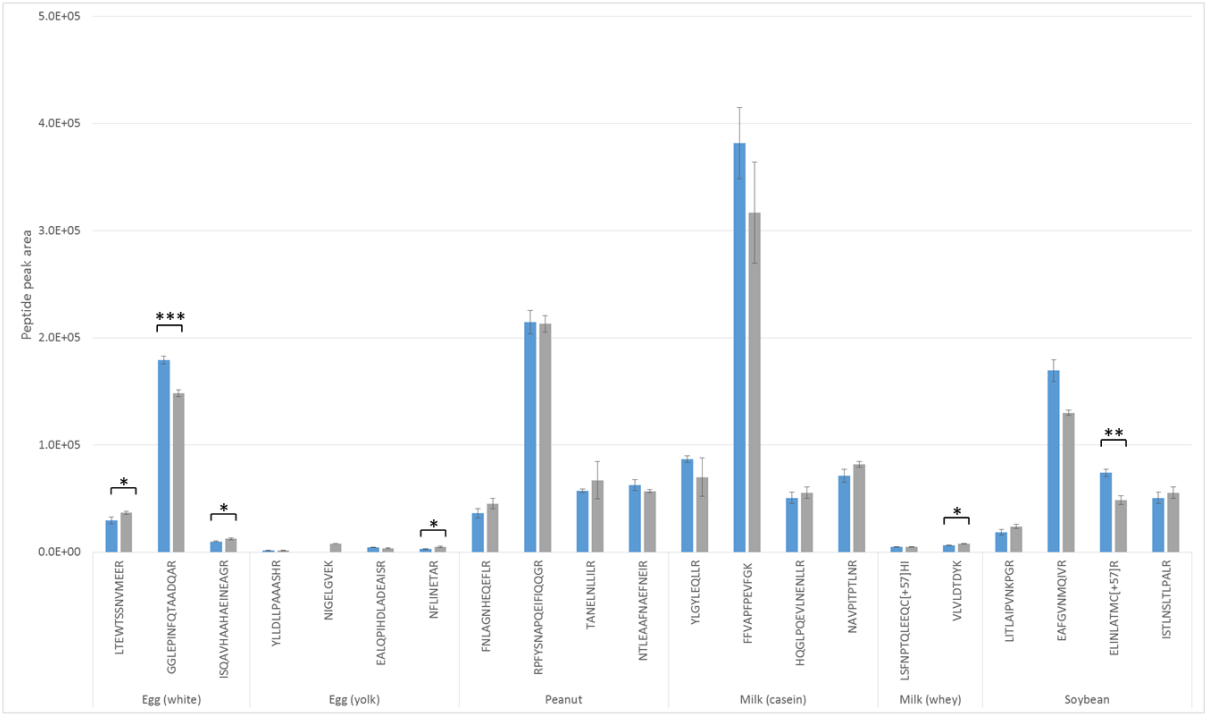

Figure 48: *Effect of washing the SPE C18 cardridge with 0.1% formic acid (blue columns) or with ACN/0.1% formic acid (95:5 v/v) (gray columns) on the peak areas for milk, egg, soy, and peanut peptides in incurred cookies (180 °C – 18 min) containing 10 mg milk proteins, 60 mg egg proteins, 100 mg soy proteins and 50 mg peanut proteins per kg. Results are expressed as means ± 1 S.D and statistical analysis was performed with Student's test (n=3 independent extractions): p value: < 0.001 (***), < 0.01 (**), < 0.05 (*).*

Addition of an organic solvent failed to yield a cleaner extract and decreased the peak areas for two peptides. We thus kept 0.1% formic acid as the washing solvent used to flush out impurities.

II.III Selecting the evaporation temperature

After peptide elution, 30 µl of DMSO were added to the collecting tube to avoid dryness before evaporation in a water bath under a nitrogen flow. As a routine method should be as short as possible, we aimed to reduce the analysis time by increasing the temperature of water bath from 40 to 60 °C under a nitrogen flow. It has been shown in the literature that avoiding complete dryness increases peptide recovery (Rosenthal et al., 2011). Consequently, a final concentration of 2 % DMSO in the samples avoids dryness and guarantees the homogeneity of samples in routine laboratories (Rosenthal et al., 2011). Moreover, the DMSO was also used by laboratories to solubilize synthetic peptides and to enhance electrospray ionization of peptides due to it low surface tension leading to the generation of more ions and increase in peptide peak areas (Breiteneder et al., 2004 b; Judák et al., 2017).

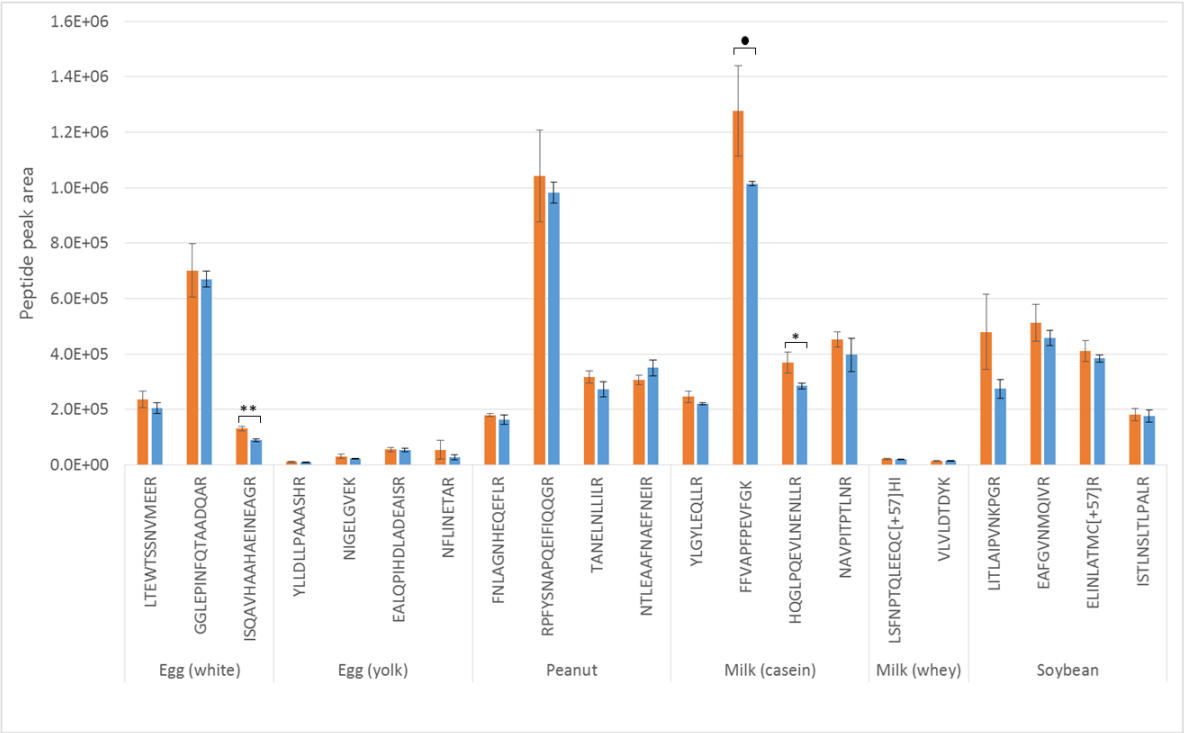

Figure 49 : *Effect of the evaporation temperature (40 or 60 °C, orange and blue columns, respectively) on the mean of peak areas for milk, egg, soy, and peanut peptides in incurred cookies (180 °C – 18 min) containing 20 mg milk proteins, 120 mg egg proteins, 200 mg soy proteins, 100 mg of peanut proteins per kg. Results are expressed as means ± 1 S.D and statistical analysis was performed with Student's test (n=3 independent extractions): p value: < 0.01 (**), < 0.05 (*), < 0.1 (●).*

The peak areas for 3 of the 21 peptides (ISQAVHAAHAEINEAGR (egg), FFVAPFPEVFGK and HQGLPQEVLNENLLR (milk)) were significantly smaller when evaporation was performed at 60 °C than when it was performed at 40 °C. This difference could be explained by a common phenomenon of peptide adsorption onto plastic which increases with a higher temperature (Niu et al., 1998). Although the evaporation time was shorter, as high sensitivity is the main goal, we retained 40 °C as the evaporation temperature **(Figure 49)**.

II.IV Selecting the elution buffer

The last factor tested to improve the purification step was the elution of peptides from the SPE cartridge. The percentage of organic solvent was increased in order to decrease the time of evaporation and potentially to improve the elution of hydrophobic peptides such as FFVAPFPEVFGK, YLGYLEQLLR, and TANELNLLILR.

Two elution conditions were tested: the first one with 1.8 ml ACN/0.1% formic acid (30:70 v/v) followed by 3.6 ml ACN/0.1% formic acid (80:20 v/v) and the second one with 6 ml ACN/0.1% formic acid (80:20 v/v).

After sample analysis, the peak areas of 18 of the 21 peptides were significantly higher when the elution buffer contained a higher volume of ACN (6 ml ACN/0.1% formic acid (80:20 v/v) (**Figure 50**).

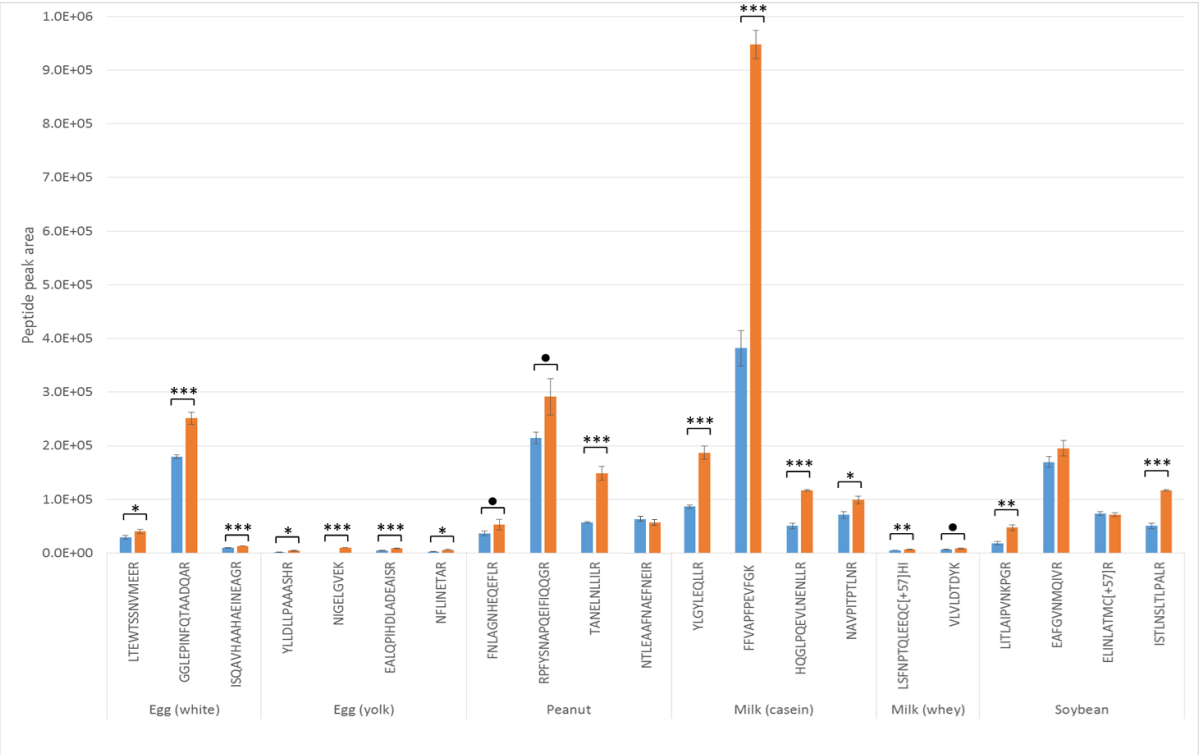

Figure 50: *Effect of the SPE elution buffer (ACN/0.1% formic acid: (30/70 v/v) 3.6 mL and 1.8 mL (80/20 v/v) in blue or 6mL (80/20 v/v) in orange on the mean of peptide peak areas for milk, egg, soy and peanut peptides in incurred cookies (180 °C – 18 min) containing 10 mg of milk proteins, 60 mg of egg proteins, 100 mg of soy proteins and 50 mg of peanut proteins per kg. Results are expressed as means ± 1 S.D and statistical analysis was performed by a Student's test (n=3 independent extractions): p value: < 0.01 (**), < 0.05 (*), < 0.1 (●).*

Considering the improvement of the peptide peak areas, the chosen eluent was ACN/0.1% formic acid (80:20 v/v). Moreover, the evaporation time was shortened with a percentage of ACN set at 80% instead of 63% as used previously.

III Selection of marker peptides for tree nuts

Target peptide selection is described in publication (Planque et al., 2017 a). First, target proteins were selected in Uniprot and a list of peptides and transitions was then generated by Skyline.

Fine ground tree nuts were extracted individually at 1 mg protein/ml in 200 mM TRIS-HCl, pH 9.2, 2 M urea and diluted to 0.1 mg/ml in 200 mM NH_4HCO_3. Trypsin digestion was carried out without any further purification and the digestion products were analyzed by UHPLC-MS/MS.

Afterwards, selection of intensely processed peptides was performed on cookies (180 °C-18 min) containing almond, walnut, pecan nut, pistachio, hazelnut, and cashew at 400 mg proteins per kg. Cookie proteins were extracted in 200 mM Tris-HCl pH 9.2, 2M urea and purified before analysis by UHPLC-MS/MS (**Table 7**).

		Almond	**Hazelnut**	**Walnut**	**Cashew**	**Pecan nut**	**Pistachio**
1 Uniprot	Proteins	11	18	4	7	19	14
2 In-silico digestion	Peptides	227	136	165	291	354	466
	Transitions	4400	2881	3413	5780	7160	9421
3 Raw ingredients analysis	Peptides	49	47	42	57	194	168
	Transitions	299	256	181	241	956	774
4 processed matrices	Peptides	22	20	27	22	132	86
	Transitions	92	74	104	85	557	371

Table 7: *Numbers of proteins, peptides, and transitions for almond, hazelnut, walnut, cashew, pecan nut, and pistachio, for the final selection of marker peptides according to four criteria.*

Marker peptides were determined by UHPLC-MS/MS in cookie, sauce, chocolate, and ice cream. Only specific peptides giving high-intensity transitions and detectable in all matrices were conserved (**publication Table 1 a-b**).

Arising from these optimizations, a publication for the detection, by UHPLC-MS/MS, of marker peptides from milk, egg, soy, peanut and tree nuts (almond, hazelnut, walnut, cashew, pecan nut and pistachio) in processed (cookies and tomato sauce) and unprocessed (chocolate and ice cream) foodstuff was published.

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Liquid chromatography coupled to tandem mass spectrometry for detecting ten allergens in complex and incurred foodstuffs

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Soy

ABSTRACT

Food allergy is a considerable heath problem, as undesirable contaminations by allergens during food production are still widespread and may be dangerous for human health. To protect the population, laboratories need to develop reliable analytical methods in order to detect allergens in various food products. Currently, a large majority of allergen-related food recalls concern bakery products. It is therefore essential to detect allergens in unprocessed and processed foodstuffs. In this study, we developed a method for detecting ten allergens in complex (chocolate, ice cream) and processed (cookie, sauce) foodstuffs, based on ultra-high performance liquid chromatography coupled to tandem mass spectrometry (UHPLC–MS/MS). Using a single protocol and considering a signal-to-noise ratio higher than 10 for the most abundant multiple reaction monitoring (MRM) transition, we were able to detect target allergens at 0.5 mg/kg for milk proteins, 2.5 mg/kg for peanut, hazelnut, pistachio, and cashew proteins, 3 mg/kg for egg proteins, and 5 mg/kg for soy, almond, walnut, and pecan proteins. The ability of the method to detect 10 allergens with a single protocol in complex and incurred food products makes it an attractive alternative to the ELISA method for routine laboratories.

1. Introduction

Currently, food contamination with allergens during production remains a challenge for the food industry, especially considering the increasing number of allergic consumers [1]. After an adverse reaction to a food, the allergen must be excluded from the diet of allergic individuals, but involuntary contaminations of the food chain make this avoidance nearly impossible [2]. Allergic patients can be sensitized to one or more proteins and each protein contains several allergenic epitopes that might differ between various individuals. To protect allergic people, various legislative texts (directives 2007/68/EC and 2000/13/EC, regulation 2011/1169/EC) require that consumers be well informed, via the product label, of the presence of 14 allergens when they are incorporated in the recipe [3,4]. These texts, however, take no account of accidental contaminations, which can also cause severe allergic reactions. Hence, the food industry has widely used precautionary allergen labeling (PAL): "may contain. . .". Yet the undeclared presence of allergens in food products is still the main cause of food recalls in developed countries (42% to 92% depending on the country) [5,6]. The lack of association between PAL and the presence of allergens also leads allergic customers to pay little attention to PAL [7]. To protect the population of allergic people, laboratories need to develop reliable, sensitive methods for the detection and quantification of allergens in food products. An obstacle is the lack of legal recommendations for food allergen thresholds, which complicates the determination of sensitivity thresholds to be achieved and the correct way to express the results of analyses. The European Academy of Allergy and Clinical Immunology (EAACI) and the Allergen Bureau (with its Voluntary Incidental Trace Allergen Labelling (VITAL) system) have established eliciting doses (EDs) for the protection of at least 95% of allergic people [8–10]. Although these referential levels have no regulatory status, laboratories and food authorities use them as indicative thresholds to support decisions, for example in the case of food recalls. According to EAACI/VITAL, the target analytical sensitivity threshold (expressed per kg) should be lower than 0.75 mg for egg proteins, 2.5 mg for milk or tree nut proteins, 5 mg for peanut proteins, 25 mg for soybean proteins, and 50 mg for cashew proteins (portion size: 40 g).

Currently, many methods are available for detecting allergens in foods such as in wine [11–15], chocolate [16–19], and cookies

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[20–23]. Usually, however, the methods developed for the analysis of allergens in a specific food cannot be applied in routine laboratories because of the wide variety of food products to be tested. To overcome this limitation, some laboratories have developed methods for the detection of allergens in several food products [24–26]. A problem with both protein- and DNA-based detection methods for allergens is that the limit of detection or quantification (LOD or LOQ) is mostly determined in spiked matrices (incorporation of extracted proteins after food processing) or solvent instead of incurred matrices (incorporation of raw ingredients before food processing) [27,28]. The determination of LOQs by spiking leads to overestimating method sensitivity [29]. Hence, even though the sensitivities estimated by these methods can be lower than the relevant EAACI/VITAL thresholds (for example, LODs for mass spectrometry of 0.1 mg milk proteins, 0.3 mg egg proteins, and 1 mg soy proteins per kg cookie) [30], such levels of sensitivity might not be achieved with real samples, especially processed foods. Several studies have indeed shown a major decrease in the sensitivity of allergen detection in processed food products [31–33]. It is thus crucial to develop reliable methods for detecting allergens in processed products.

The improvement of sample preparation steps and the high sensitivity of mass spectrometry have allowed impressive progress in terms of detection sensitivity in processed products. Mass spectrometry method must be able to detect specific peptides coming from one or several proteins with a high sensitivity. The target proteins must be abundant, stable to the thermal process and specific for the allergen, but they don't need to be allergenic. A study by Pöpping and collaborators (2012) was the first to analyze 10 allergens simultaneously with high sensitivity in processed bread (60 min–200 °C). These authors used an ultrafiltration purification allowing LODs of 3 mg soluble proteins for almond, 5 for milk and hazelnut, 11 for peanut, 24 for soy, 42 for egg, and 70 for walnut [34]. A recent report describes a size exclusion column (SEC) purification step used before analysis of 5 allergens in incurred cookies (12 min −200 °C) by UHPLC–MS/MS [35]. The LODs, initially expressed in mg whole allergens per kg, were converted to mg proteins per kg by using VITAL conversion factors. They were 2.5 mg per kg for milk, 4.3 for egg, 3.2 for soy, 3.3 for peanut, and 1.1 for hazelnut.

We have previously developed a sensitive method for the detection of four allergens in complex and processed matrices (ice cream, sauce (95 °C–45 min), cookie (180 °C–18 min), and chocolate) [36]. This method uses a solid phase extraction (SPE) purification prior to UHPLC–MS/MS analysis. To the best of our knowledge, it is still the most sensitive method for detecting these allergens in processed matrices, with LOQs (S/N > 10) of 0.5 mg milk proteins, 2.5 mg peanut proteins, 5 mg soy proteins, and 3.4 mg egg proteins per kg food. Here we have extended this approach, applying our method to additional common allergens that must be declared on food labels. The ultimate goal was to develop a sensitive screening method for routine laboratories [36]. Given the prevalence of allergic reactions to tree-nut antigens, we prioritized this allergen category by including six tree nuts [37].

2. Materials and methods

2.1. Materials and reagents

Peanut butter (NIST 2387 22.2% protein), milk powder (NIST1549a 25.64% protein), whole egg (NIST 8445 48% protein), and soy flour (NIST 3234 53.37% protein) were from the National Institute of Standards and Technology (NIST) (Gaithersburg, Maryland, USA). Tree nuts (almonds, cashews, pecan nuts, hazelnuts, walnuts, and pistachios) were purchased from a local store before being finely ground under liquid nitrogen. Tris(hydroxymethyl)aminomethane (TRIS), urea, dimethyl sulfoxide (DMSO), DL-dithiothreitol (DTT), iodoacetamide (IAA), ammonium bicarbonate, and trypsin from bovine pancreas (T8802) were from Sigma-Aldrich (Bornem, Belgium). Acetic acid was from Acros Organics (Geel, Belgium) and hydrochloric acid was obtained from Fisher Chemical (Loughborough, UK). Sep-pak C18 solid phase extraction (SPE) columns (6 cc, 500 mg – WAT043395) were used for peptide purification and enrichment and purchased from Waters (Milford, Massachusetts, USA). Acetonitrile, 2-propanol, methanol (ULC–MS grade), and formic acid were obtained from Biosolve (Valkenswaard, the Netherlands). The labeled peptides TANELNLLI**L** [$^{13}C_6$ ^{15}N]R-OH, FFVAPFPEVFG**K** [$^{13}C_6$ $^{15}N_2$]-OH, GGLEPIN**F** [D_5] QTAADQAR-NH2, and EAFG**V** [D_8] NMQIVR-OH were purchased from Eurogentec (Seraing, Belgium).

2.2. Preparation of non-contaminated matrices

Chocolate, tomato sauce, cookie, and ice cream were selected as targeted food products for the detection of allergens. Cookie dough was prepared by mixing flour (53.4%), sugar (15.2%), oil (16.1%), water (14.8%), NaCl (0.3%), ammonium bisulfate (0.1%), and sodium bicarbonate (0.1%) with a blender. Ice cream was prepared by mixing banana (59.0%), coco milk (29.6%), sugar (11.0%), and lemon juice (0.4%) with a blender. Allergen-free chocolate (containing cacao (45%), sugar (35%), and rice powder (20%)) and tomato sauce (containing mainly tomatoes (75%) and a mixture of onions, carrots, and celery (15%)) were purchased from a local store and finely ground.

2.3. Preparation of incurred materials

Raw food allergens were combined with the flour (cookie), coco milk (ice cream), crushed chocolate, and sauce to obtain a theoretical calculated allergen protein concentration of 20 mg/kg in the milk matrix, 100 mg/kg for peanut and tree nuts, 120 mg/kg for egg, and 200 mg/kg for soybean (n = 3). Crushed chocolate containing allergens was heated in a water bath at 40 °C for 20 min and frozen at −80 °C before being finely ground. After incorporation of allergens, sauce batches were warmed at 95 °C for 45 min. Mechanical grinding (Robot-Coupe, Blixer 4 V.V.) was performed for 3 min to achieve a homogenous distribution of food allergens in ice cream and cookie, before freezing the ice cream at −20 °C. Serial dilutions of contaminated matrices were prepared by mixing non-contaminated and contaminated matrices to reach the following intermediate levels of food allergens: 0, 0.1, 0.5, 1, 2.5, 5, 10, and 20 mg/kg for milk proteins, 0, 0.5, 2.5, 5, 12.5, 25, 50 and 100 mg/kg for tree nut and peanut proteins, 0, 0.6, 3, 6, 15, 30, 60 and 120 mg/kg for egg proteins, and 0, 1, 5, 10, 25, 50 100 and 200 mg/kg for soy proteins. Eight 40-g cookies 7 cm in diameter were prepared per intermediate level of contamination and per independent replicate (n = 3). In each batch, the cookies were baked at 180 °C for 18 min and then finely milled in a blender.

2.4. Preparation of spiked solution

On the basis of NIST protein contents, a milk solution at 0.1 mg/mL was prepared in extraction buffer (200 mM TRIS-HCl pH 9.2, 2 M urea). This solution was used to spike dietary supplements and enzyme samples at 1 mg/kg and 2.5 mg/kg prior to applying the protocol.

2.5. Sample preparation for UHPLC–MS/MS analysis

2.5.1. Extraction and enzymatic digestion protocol

The protocol applied was as described in [36] with slight modifications. Three grams of matrix was weighed into a 50 mL

Table 1a
Selected peptides and multiple reaction monitoring (MRM) parameters for the analysis of soy, milk, egg, and peanut allergens by mass spectrometry (UHPLC–MS/MS). A BLAST search was performed to ensure peptide specificity (supplementary data 1).

Allergen	Protein	Peptide	Retention time in sauce (min)	Precursor (charge state) (*m*/*z*)	Product ion (fragments)	Collision energy (eV)
Soy	Glycinin P04347 Glycinin A3B4	ISTLNSLTLPALR	14.2	699.9 (++)	984.6 (y9)	23
					870.5 (y8)	25
					783.5 (y7)	25
	Glycinin G2 P04405 Gly m6	EAFGVNMQIVR	12.3	632.3 (++)	859.5 (y7)	18
					760.4 (y6)	17
					646.4 (y5)	22
	2S albumin P19594 Gly m 2S albumin	ELINLATMC[+57]R	12.4	610.8 (++)	865.4 (y7)	21
					751.4 (y6)	21
					638.3 (y5)	17
	Glycinin G1 P04776 Gly m6	VFDGELQEGR	8.7	575.3 (++)	788.4 (y7)	20
					602.3 (y5)	20
					789.4 (b7)	20
	Beta-conglycinin P13916 Gly m Bd60K	LITLAIPVNKPGR	12.4	464.6 (+++)	767.5 (y7)	15
					583.4 (y11)	9
					476.3 (y9)	11
Milk	Casein αS1 P02662 Bos d 8	HQGLPQEVLNENLLR	12.2	587.3 (+++)	871.5 (y7)	17
					758.4 (y6)	16
					436.2 (b4)	17
		FFVAPFPEVFGK	16.6	692.9 (++)	991.5 (y9)	18
					920.5 (y8)	18
					676.4 (y6)	28
		YLGYLEQLLR	15.5	634.4 (++)	934.5 (y7)	21
					771.5 (y6)	20
					658.4 (y5)	21
	Casein αS2 P02663	NAVPITPTLNR	10.2	598.3 (++)	911.5 (y8)	17
					456.3 (y8)	14
					285.2 (b3)	12
	P0β-lactoglobulin P02754 Bos d 5	VYVEELKPTPEGDLEILLQK	14.0	771.8 (+++)	912.0 (y16)	19
					790.9 (y14)	19
					627.9 (y11)	20
		VLVLDTDYK	11.00	533.3 (++)	853.4 (y7)	15
					754.4 (y6)	14
					641.3 (y5)	16
		LSFNPTQLEEQC[+57]HI	12.8	858.4 (++)	1254.6 (y10)	26
					928.4 (y7)	27
					627.8 (y10)	27
Egg	Ovalbumin P01012 Gal d 2	GGLEPINFQTAADQAR	11.6	844.4 (++)	1331.7 (y12)	26
					1121.5 (y10)	28
					666.3 (y12)	25
		LTEWTSSNVMEER	10.4	791.4 (++)	1052.5 (y9)	31
					951.4 (y8)	23
					864.4 (y7)	23
		ISQAVHAAHAEINEAGR	6.4	887.5 (++)	1138.6 (y11)	33
					1067.5 (y10)	33
					996.5 (y9)	32
	Vitellogenin-2 P02845	EALQPIHDLADEAISR	11.8	593.3 (+++)	761.4 (y7)	19
					690.3 (y6)	15
					668.8 (y12)	15
		NIPFAEYPTYK	11.7	671.8 (++)	1115.5 (y9)	15
					508.3 (y4)	16
					558.3 (y9)	29
		NIGELGVEK	9.0	479.8 (++)	731.4 (y7)	12
					674.4 (y6)	10
					545.3 (y5)	19
	Vitellogenin-1 P87498	YLLDLLPAAASHR	14.1	480.6 (+++)	709.4 (y7)	15
					582.3 (y11)	10
					355.2 (y7)	14
Peanut	Cupin Q8LKN1 Ara h 3/4	RPFYSNAPQEIFIQQGR	11.4	684.4 (+++)	748.4 (y6)	20
					608.3 (y10)	19
					836.4 (b7)	17
		FNLAGNHEQEFLR	10.7	525.6 (+++)	692.4 (y5)	20
					600.8 (y10)	13
					565.3 (y9)	14
	Cupin Q647H4 Ahy-1	TANELNLLILR	14.8	635.4 (++)	983.6 (y8)	21
					854.6 (y7)	20
					741.5 (y6)	22
	Conglutin 7 Q6PSU2 Ara h2	NLPQQC[+57]GLR	7.4	543.3 (++)	858.4 (y7)	13
					633.3 (y5)	22
					429.7 (y7)	16

Table 1b
Selected peptides and multiple reaction monitoring (MRM) parameters for the analysis of walnut, pecan, almond, cashew, hazelnut and pistachio allergens by mass spectrometry (UHPLC–MS/MS). A BLAST search was performed to ensure peptide specificity (supplementary data 1).

Allergen	Protein	Peptide	Retention time in sauce (min)	Precursor (charge state) (*m*/*z*)	Product ion (fragments)	Collision energy (eV)
Walnut	Vicilin-like protein Q9SEW4 Jug r 2	ATLTLVSQETR	9.5	609.8 (++)	620.3 (y5)	19
					719.4 (y6)	22
					832.5 (y7)	21
	Albumin seed storage protein P93198 Jug r 1	GEEMEEMVQSAR	9.1	698.3 (++)	1080.5 (y9)	24
					949.4 (y8)	22
					820.4 (y7)	22
Walnut & pecan nuts	Vicilin-like protein Q9SEW4 Jug r 2 7S vicilin B3STU7 Car i 2	LLQPVNNPGQFR	10.2	691.9 (++)	1028.5 (y9)	22
					604.3 (y5)	25
					514.8 (y9)	22
		VFSNDILVAALNTPR	14.5	815.5 (++)	1067.7 (y10)	26
					954.6 (y9)	26
					841.5 (y8)	26
Pecan nuts	7S vicilin B3STU4 Car i 2	QVESYFVPMER	11.1	692.8 (++)	532.3 (y4)	23
					778.4 (y6)	24
					941.5 (y7)	22
		ATLTFVSQER	9.6	576.3 (++)	866.4 (y7)	21
					765.4 (y6)	16
					618.3 (y5)	18
		NFLAGQNNIINQLER	12.8	582.0 (+++)	659.4 (y5)	19
					772.4 (y6)	18
					885.5 (y7)	20
		LVGFGINGK	10.8	452.8 (++)	692.4 (y7)	12
					635.4 (y6)	14
					488.3 (y5)	13
Almond	Prunin Q43607 Pru du 6	GNLDFVQPPR	10.7	571.8 (++)	743.4 (y6)	19
					596.4 (y5)	14
					369.2 (y3)	20
		ALPDEVLANAYQISR	13.0	830.4 (++)	1035.6 (y9)	30
					922.5 (y8)	32
					851.4 (y7)	27
		YNRQETIALSSSQQR	7.3	594.3 (+++)	805.4 (y7)	21
					692.3 (y6)	20
					605.3 (y5)	16
		QETIALSSSQQR	7.7	674.3 (++)	876.5 (y8)	26
					805.4 (y7)	27
					692.3 (y6)	27
	Pru2 protein Q43608 Pru du 6	TDENGFTNTLAGR	9.3	698.3 (++)	936.5 (y9)	25
					879.5 (y8)	25
					732.4 (y7)	23
Cashew	Allergen Ana o 2 Q8GZP6 Ana o 2	C[+57]AGVALVR	8.7	423.2 (++)	614.4 (y6)	13
					557.4 (y5)	15
					458.3 (y4)	14
		ADIYTPEVGR	9.1	560.8 (++)	821.4 (y7)	19
					658.4 (y6)	18
					557.3 (y5)	17
		AMTSPLAGR	8.4	452.2 (++)	701.4 (y7)	13
					600.3 (y6)	15
					513.3 (y5)	15
	2S albumin Q8H2B8 Ana o 3	ELYETASELPR	9.7	654.3 (++)	902.5 (y8)	21
					773.4 (y7)	22
					672.4 (y6)	20
Hazenut	11S globulin- like protein Q8W1C2 Cor a 9	LNALEPTNR	7.9	514.3 (++)	800.4 (y7)	15
					616.3 (y5)	14
					487.3 (y4)	15
		ADIYTEQVGR	7.9	576.3 (++)	852.4 (y7)	20
					689.4 (y6)	19
					588.3 (y5)	16
		INTVNSNTLPVLR	11.5	720.9 (++)	1013.6 (y9)	25
					899.5 (y8)	27
					812.5 (y7)	23
		QGQVLTIPQNFAVAK	12.4	807.5 (++)	1088.6 (y10)	27
					987.6 (y9)	25
					874.5 (y8)	23
		TNDNAQISPLAGR	8.8	678.8	841.5 (y8)	22
					713.4 (y7)	22
					513.3 (y5)	21
		ALPDDVLANAFQISR	14.7	815.4 (++)	1019.6 (y9)	28
					906.5 (y8)	31
					723.4 (y13)	19
Pistachio	2S albumin B7P072 Pis v 1	LQELYETASELPR	11.2	774.9 (++)	1178.6 (y10)	26
					1065.5 (y9)	28
					902.5 (y8)	24
	11S globulin precusor B7SLJ1 Pis v 5	ITSLNSLNLPILK	15.0	713.4 (++)	1011.6 (y9)	21

Table 1b (*Continued*)

Allergen	Protein	Peptide	Retention time in sauce (min)	Precursor (charge state) (*m*/*z*)	Product ion (fragments)	Collision energy (eV)
					897.6 (y8)	21
					470.3 (y4)	22
		AMISPLAGSTSVLR	12.6	701.9 (++)	1000.6 (y10)	23
					790.4 (y8)	28
					719.4 (y7)	25
	11S globulin B2KN55 Pis v 2	VTSINALNLPILR	15.1	712.4 (++)	838.6 (y7)	22
					498.3 (y4)	24
					1023.6 (b10)	23
	11S globulin precusor B7P073 Pis v 2	ALPLDVIK	12.5	434.8 (++)	684.4 (y6)	10
					474.3 (y4)	17
					342.7 (y6)	11

polypropylene tube and spiked with 100 μL internal standard solution containing FFVAPFPEVFGK [$^{13}C_6$ $^{15}N^2$]-OH at 4 μg/mL and EAFGV [D_8] NMQIVR-OH, TANELNLLI**L** [$^{13}C_6$ ^{15}N]R-OH, and GGLEPIN**F** [D_5] QTAADQAR-NH2 at 7 μg/mL in 0.1% formic acid. Samples were extracted with 30 mL of 200 mM TRIS-HCl pH 9.2, 2 M urea by shaking at 20 °C for 30 min (Agitelec, France) prior to ultrasonic treatment at 4 °C for 15 min. Afterward, the samples were centrifuged at 4660*g* for 10 min at 4 °C. Extracted proteins contained in 10 mL supernatant were diluted with 10 mL digestion buffer (200 mM ammonium bicarbonate). Protein reduction was performed by incubating at room temperature (20 °C) for 45 min with 1 mL of 200 mM DTT. Protein alkylation was performed by incubation for 45 min in the dark with 400 mM IAA. Enzymatic digestion was performed by adding 1 mL tryspin (1 mg/mL in 50 mM acetic acid) and incubating at 37 °C for 1 h. The digestion reaction was stopped by addition of 300 μL of 20% formic acid. At this step, the sample could be kept at −20 °C prior to clean-up the following day. Sample extracts were finally centrifuged at 4660*g* for 5 min at 20 °C prior to SPE clean-up.

2.5.2. Sample purification protocol

After enzymatic digestion, the peptides were concentrated and purified on C18 SPE cartridges. Conditioning was done with acetonitrile (18 mL) followed by 0.1% formic acid (18 mL). The column was loaded with sample (20 mL) and washed with 0.1% formic acid (18 mL). DMSO (30 mL) was added to the collector tube before elution with acetonitrile/0.1% formic acid (80/20, v/v) (6 mL). Under a nitrogen flow at 40 °C, the samples were evaporated and then the pellets were dissolved in 0.1% formic acid/acetonitrile (95/5, v/v) (600 μL). Extracts were centrifuged at 4660*g* for 5 min at 10 °C, transferred to a microtube, and centrifuged again for 5 min at 11754 g and 4 °C. The samples were then analyzed by UHPLC–MS/MS.

2.5.3. UHPLC–MS/MS parameters for peptide analysis

An Acquity system (Waters – Milford, Massachusetts, USA) with a thermal autosampler (set at 10 °C) and a column compartment (set at 50 °C) were used. After injection of a 20-μL sample, peptide separation was performed on a C18 Acquity BEH130 Waters column (2.1 × 150 mm) at 0.2 mL/min. A gradient was applied for 26 min (solvent A: 0.1% formic acid – solvent B: acetonitrile plus 0.1% formic acid) to separate the targeted peptides (0–3 min: 92% A; 3–18 min: 92% to 58% A, 18.0–18.1 min: 58% to 15% A; 18.1–22.5 min: 15% A; 22.5–22.6 min: 15% to 92% A, 22.6–26 min: 92% A). To avoid carry-over (peaks attributed to the previously analyzed sample may be observed in the subsequent chromatograms), the UPLC column was flushed out with methanol/2-propanol/acetonitrile/water (25/25/25/25 v/v/v/v) for 15 min before returning to the initial conditions (92% solvent A) for 5 min. Detection was performed with a Waters Xevo TQS triple quadrupole system in the MRM and positive electrospray modes. The desolvation temperature was set at 500 °C and the nitrogen flow at 1200 L/h. The cone nitrogen flow was set at 150 L/h, the capillary voltage at 2.0 kV, the collision gas flow at 0.12 mL/min, and the source temperature at 150 °C.

3. Results and discussion

3.1. Selection of marker peptides

The marker peptide selection procedure has been previously described in [36]. Briefly, an *in-silico* digestion with Skyline software generates a list of peptides, MRM transitions, and MS/MS parameters based on criteria selected by the user (peptide length, charge states, enzyme...). Raw ingredients and incurred cookies were then prepared and analyzed in order to select abundant, robust peptides from the list generated by Skyline. A last selection criterion was based on the allergens detected in four selected incurred matrices: sauce (thermal process and acid), ice cream (fat), chocolate (tannins), and cookie (high-thermal process). Only 3 to 5 peptides were retained on the basis of specificity and sensitivity of detection, with 3 transitions per peptide. Selected peptides belong to one or several proteins depending on the allergen of interest. The specificity and the signal intensity of peptides into the four target matrices were the main criteria used for the selection of peptides. The list of target peptides is presented in Table 1a and 1b. Finally, BLAST analyses were run to search for 100%-homologous peptides, in order to ensure peptide specificity. The selected peptides for soy, peanut, walnut, pecan nuts, cashew, hazelnut, and pistachio appeared totally specific according to BLAST analyses, but the selected peptides for almond, milk, and egg were found not to be totally restricted to one species.

For almond, 97.2% homology was found between the almond (*Prunus dulcis*) protein Pru du 6 and its peach kernel (*Prunus persica*) counterpart (BLAST Uniprot). Of the five selected peptides, only one appeared totally specific to almond TDENGFTNTLAGR (Table 1b), but as peach kernels are present only in persipan or in cases of food fraud (when almond is replaced for economic reasons), their unintentional presence should not be frequent. For this reason, the five selected peptide markers could be kept. The homology of selected peptides with other species is presented in Table 1 - complementary data.

The high degree of protein homology between animal species for egg and milk is not a problem, because in most cases the allergenic protein can trigger an allergic reaction whatever the animal of origin (e.g. yak, buffalo, goat, or sheep casein and whey proteins). Therefore, the egg and milk peptides listed in Table 1a were considered here despite the fact that they are common to different species.

Homology was found between the ovalbumin peptides and certain peptides of *Achromobacter denitrificans*, isolated frequently from soil, water, and rotten milk and eggs [38,39]). This bacterial species, not referred to in Uniprot in 2016, is not expected in fresh food products.

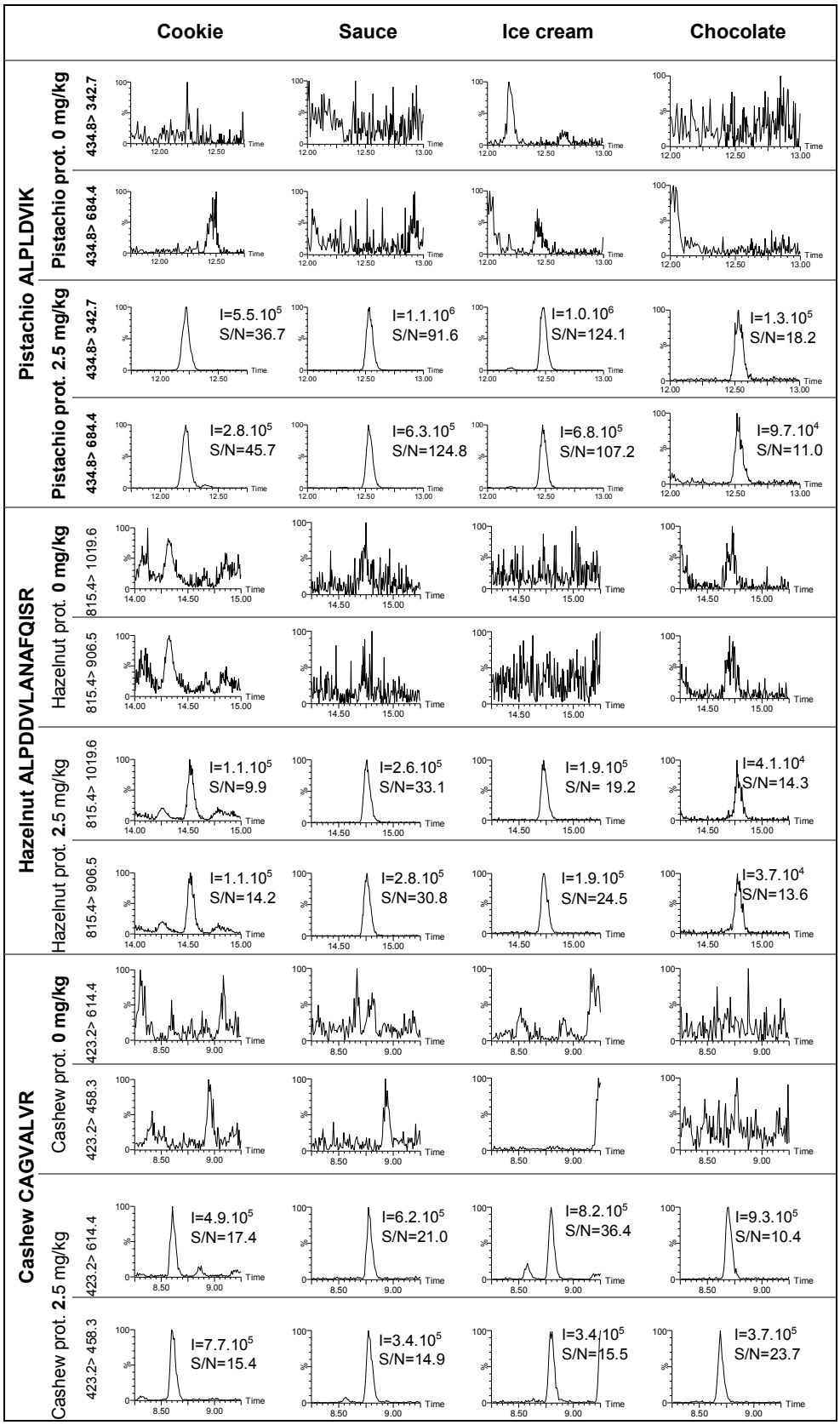

Fig. 1. Chromatograms of the two highest multiple reaction monitoring MRM transitions for the most abundant peptide of pistachio, hazelnut, cashew, almond, walnut, pecan nut, milk, egg, soy, and peanut allergens. Data for non-contaminated matrices (0 mg/kg) and for incurred matrices at the limit of quantification are presented without smoothing.

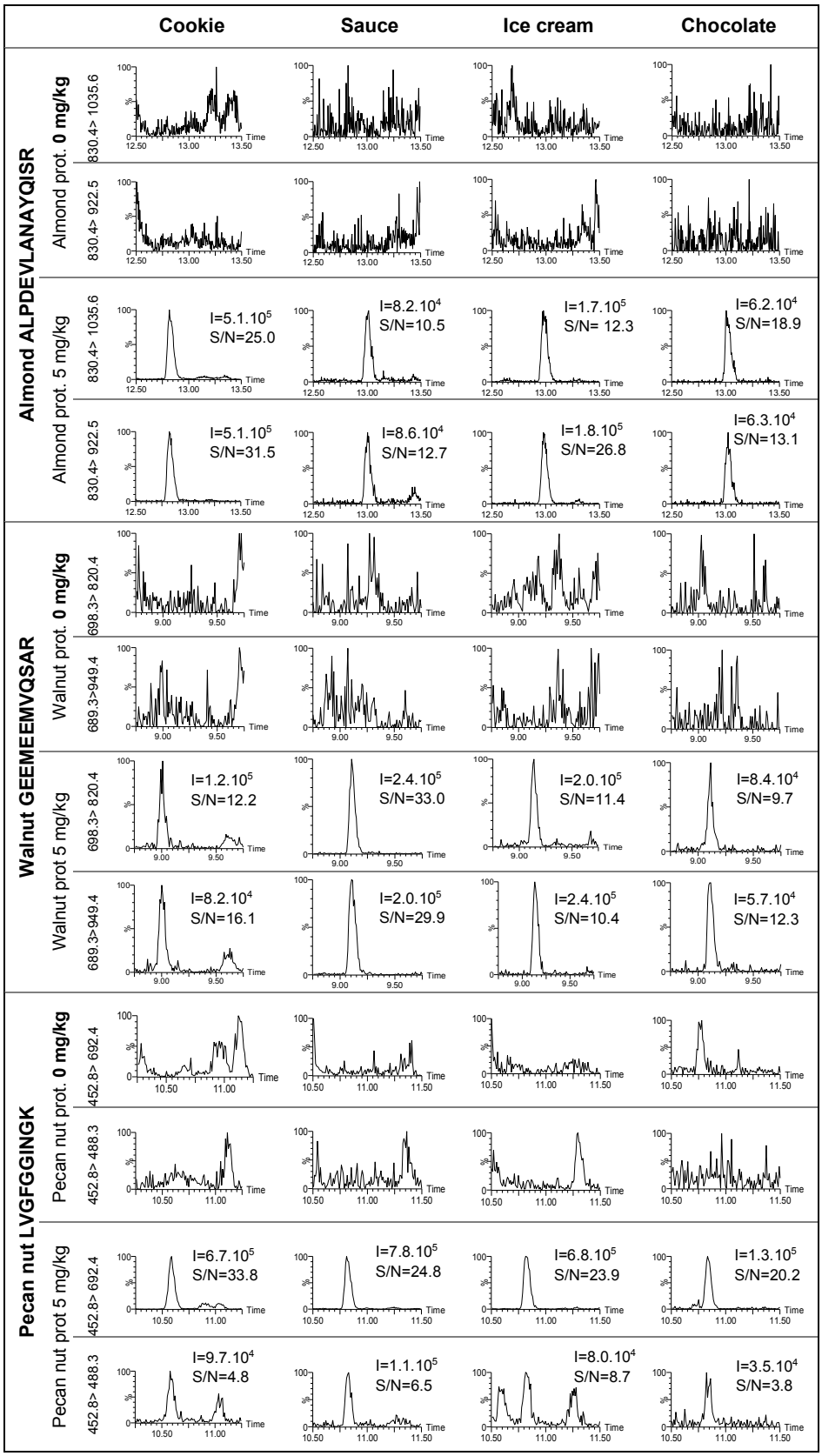

Fig. 1. (*Continued*)

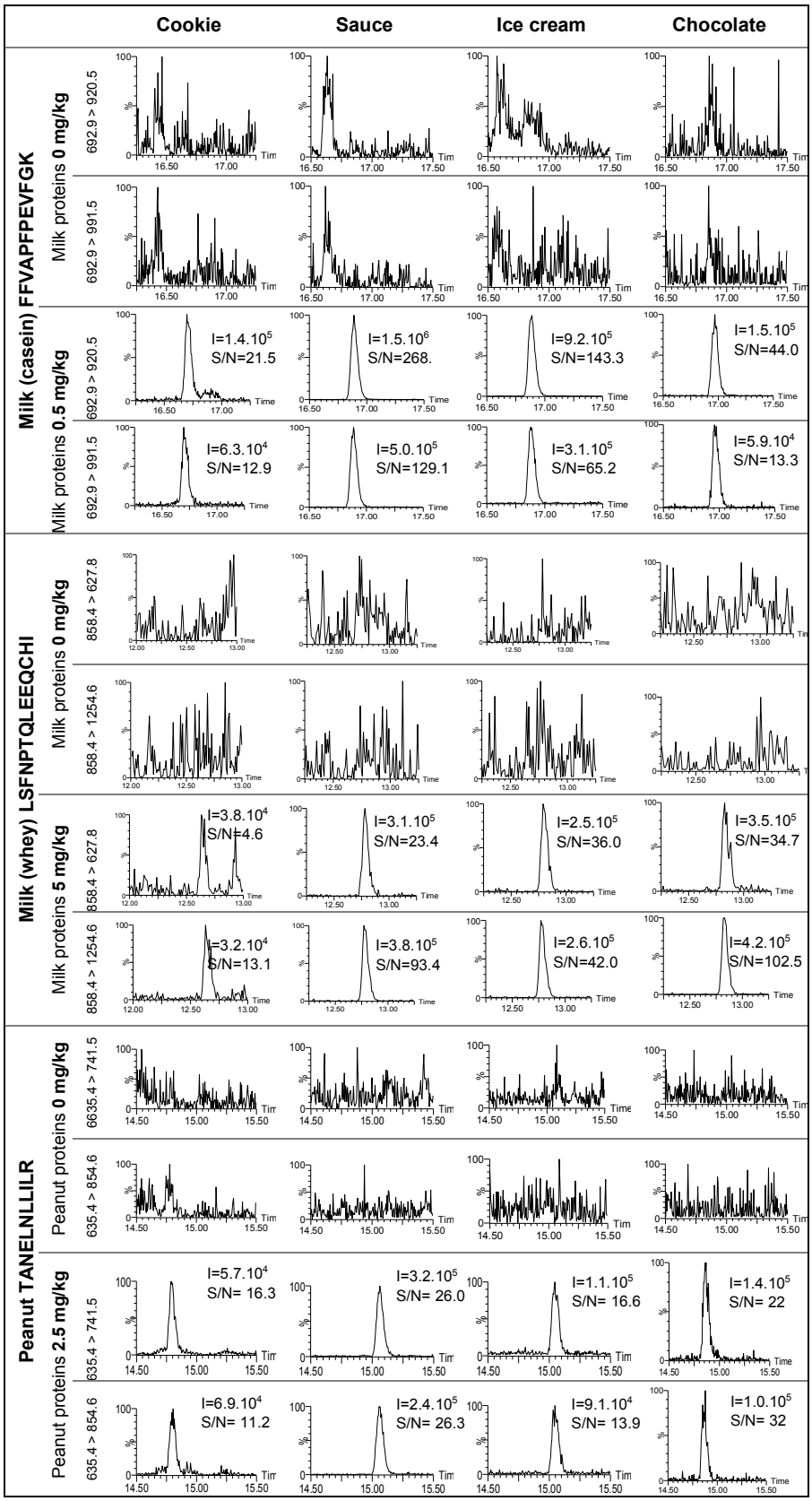

Fig. 1. (*Continued*)

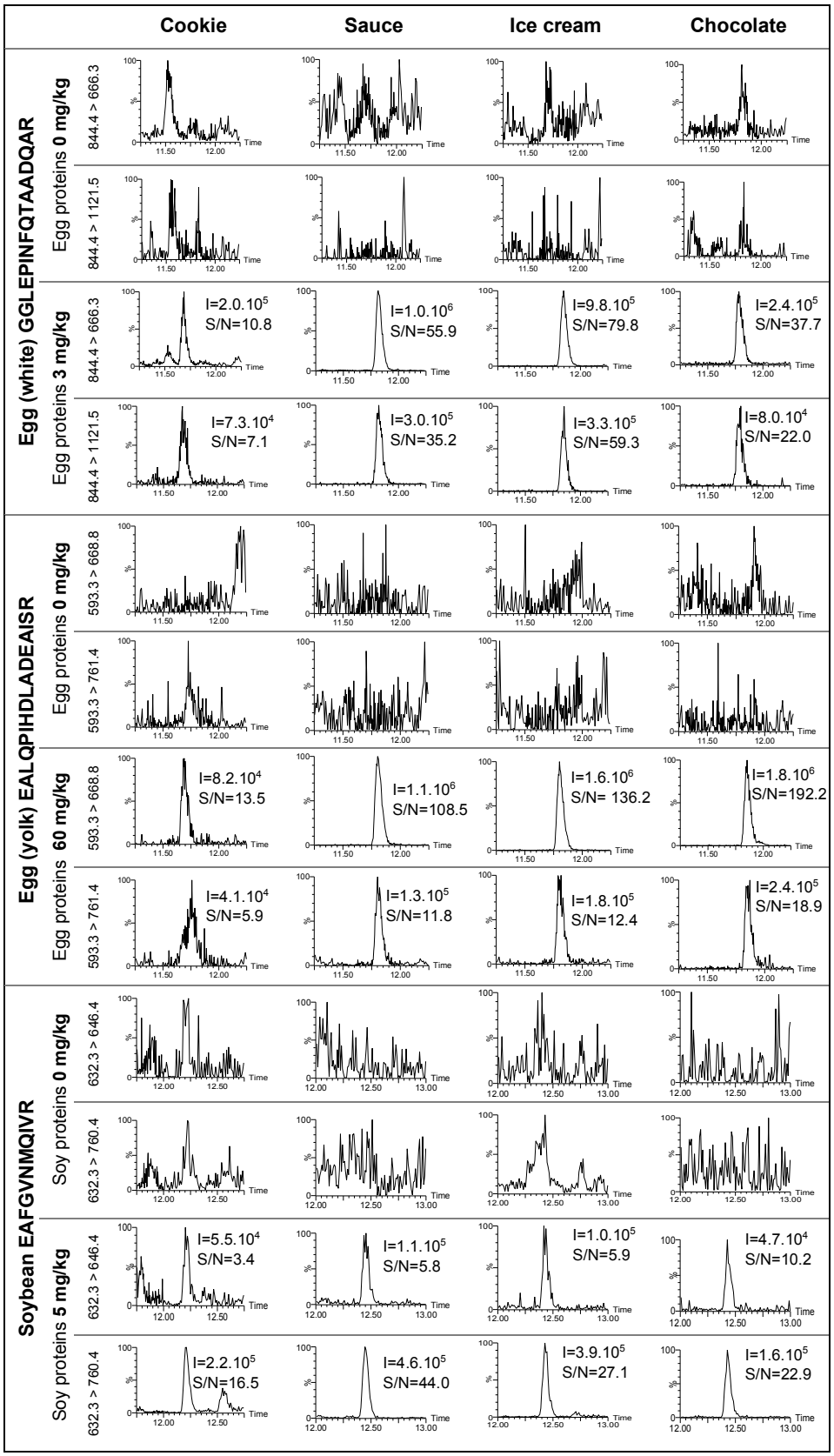

Fig. 1. (*Continued*)

In our selection, two peptides were found to be common to pecan and walnut. These peptides should be used only to confirm the presence of tree nuts in foodstuffs. They were not used here to determine the sensitivity of our method.

Once the peptides were selected, we performed sample analyses to narrow down the number of transitions, as a high number was predicted (see Tables 1a and 1b). As MRM transition quality was expected to depend on the matrix, samples containing the ten allergens prepared in four different matrices (complex and processed) were systematically analyzed, with two injections per sample. Considering the constraint that a method for routine laboratories should be efficient and rapid, for each allergen only the two to three most abundant peptides with two MRM transitions were selected, to allow the simultaneous analysis of 10 allergens in 26 instead of 52 min. The time of analysis was thus reduced. The selected peptides should be tested in more matrices (complex and processed), however, to ensure the capacity of the method to detect the target peptides.

3.2. Determination of method performances

To determine method performances, four parameters were evaluated (sensitivity, specificity, linearity, repeatability). The sensitivity and linearity were evaluated by analyzing three independent preparations for each matrix (cookie, sauce, chocolate, ice cream) at eight different concentrations. The specificity of the method was determined by analyzing the four allergen-free matrices. The repeatability, evaluated by a run relative standard deviation (RSD), was determined by analyzing six aliquots of the same foodstuff preparation.

3.2.1. Sensitivity and specificity

To ensure the applicability of the method in a routine laboratory, a limit of quantification (LOQ) per allergen, common to the four matrices, was determined for each peptide by analyzing target incurred foodstuffs. Signal-to-noise ratios (S/N) of respectively 10 and 3 for the first and second most intense MRM transitions of the targeted food allergen allowed determining the sensitivity of the method. The S/N ratio was calculated in peak-to-peak mode, without any 'ignore noise' scan, for three independent foodstuff preparations.

For each peptide, two MRM transitions in allergen-free and LOQ-contaminated matrices are presented to demonstrate the specificity and sensitivity of the method (Fig. 1–Fig. 1 Complementary data). The two peptides common to pecan and walnut proteins were not used to determine the sensitivity. The LOQs reached were: 0.5 mg milk proteins/kg for caseins, 5 mg milk proteins/kg for whey proteins, 3.0 mg egg proteins/kg for egg white, 60 mg egg proteins/kg for egg yolk, 2.5 mg/kg for peanut, cashew, pistachio, and hazelnut proteins, and 5 mg/kg for soybean, almond, walnut, and pecan nut proteins. The results obtained demonstrate the suitability of the method, since the target thresholds (VITAL/EAACI) were reached for six out of ten allergens (S/N > 10). Furthermore, the method was able to detect (S/N > 3) 0.6 mg egg proteins and 2.5 mg walnut, almond, and pecan proteins per kg (Fig. 2–complementary data). The modification introduced with respect to the initial protocol (see Materials and Methods) led to a minor decrease in sensitivity for egg yolk (60 mg instead of 30.8 mg egg proteins per kg), but fouling of the mass spectrometer and protocol time were considerably decreased, making the method suitable for routine laboratories.

3.2.2. Matrix effects and relative standard deviations (RSDs)

Three independent preparations of foodstuffs in each targeted matrix (incurred chocolate, ice cream, cookies, and sauce) were used to determine the linearity of the peptide peak area as a function of allergen concentration. Each contaminated matrix was mixed with non-contaminated matrix to obtain different concentrations of milk, egg, soy, peanut and tree nuts. Fig. 3 -complementary data shows an illustrative detailed regression analysis. The regression coefficient (R^2) was determined when at least five levels of concentration including the blank were detected (Table 2). The R^2 was higher than 0.99 for 97 linear regressions out of 151 (64.2%) and higher than 0.97 for 137 other linear regressions (90.7%). This result may be due to the difficulty of ensuring a homogeneous distribution of particles in three independent foodstuff preparations and to the lack of correction of protocol steps with internal standards. In most cases, the slopes were lower in incurred cookies and chocolate than in ice cream and sauce, probably due to high-thermal processing in the case of cookies and to the presence of tannins in chocolate. The differences in slope between matrices show major matrix effects and highlight the need to add isotope-labeled peptides to correct for matrix effects.

Relative standard deviations were calculated by analyzing, for each matrix, 6 aliquots of the same food preparation at 10 mg milk proteins, 50 mg tree nut and peanut proteins, 60 mg egg proteins, and 100 mg soy proteins per kg food. A 2016 guideline for the detection and quantification of allergens by mass spectrometry (SMPR 2016.002) [40] specifies that the RSD should be lower than 20%. Out of 198 RSDs determined for the four target matrices (Table 2), 23 (11.6%) were higher than 20%. All the presented data were obtained without internal standard correction, for lack of labeled internal standards for all peptides. Subsequently, however, for milk, egg, peanut, and soy peptides, corresponding isotope-labeled standards were introduced before extraction. For milk peptide FFVAPFPEVFGK in chocolate, correction with the corresponding labeled internal standard decreased the RSD from 30.9% to 10.3%. It should also be stressed that the SMPR.2016.002 guideline specifications were determined for spiked and not incurred samples. Moreover, important criteria are missing, for example, the guidelines do not specify the number of target peptides that must be identified to consider a sample as a positive one. In spiked samples, proteins are extracted in a solvent prior to being added to the matrices, which often leads to lower RSDs than for incurred samples. To determine the RSD of the method, allergens should be analyzed in certified incurred materials with correction by means of internal labeled standards in order to evaluate whether the guideline specifications can be reached. To obtain a higher coefficient of regression R^2 and a lower RSD in a routine method, labeled peptides should imperatively be used. This was done here in the context of real sample analysis.

3.3. Analysis of real samples with an internal labeled standard

The method was further used to detect milk in dietary supplements and enzymes (cellulase and protease). These are matrices which could not be analyzed by ELISA: despite standard addition at 1 or 2 mg milk proteins per kg, a strong matrix effect prevented their detection (data not shown).

The samples were spiked at 0, 1, or 2.5 mg milk proteins per kg (2x LOQ and 5x LOQ, respectively), together with 0.4 μg internal standard. Proteins were extracted, samples were prepared as described under Materials and Methods, and transitions of both the unlabeled and labeled FFVAPFPEVFGK milk casein peptide were sought by UHPLC–MS/MS.

Fig. 2 shows chromatograms of one MRM transition for the milk casein peptide FFVAPFPEVFGK and its associated labeled standard in two dietary supplements and two enzymes. The internal standard was detected in both supplements, but milk was not detected in dietary supplement B after standard addition of milk. Non-detection of milk was most likely due to the high content in isolated soy proteins (>20%), which probably caused at least partial inhibi-

Table 2
Linear regression of peptide peak area of the most abundant MRM transition as a function of the concentration of allergen proteins for tree nuts, egg, soy, peanut, and milk. Relative standard deviations (RSDs) were calculated by analyzing 6 times the same foodstuff preparation containing 50 mg proteins from each tree nut, 50 mg peanut proteins, 10 mg milk proteins, 60 mg egg proteins, and 100 mg soy proteins per kg of incurred sauce, ice cream, chocolate, or cookies.

			Chocolate				Cookie				Ice cream				Sauce			
Allergen	Peptide	LOQ	Slope	Intercept	R^2	RSD (%)	Slope	Intercept	R^2	RSD (%)	Slope	Intercept	R^2	RSD (%)	Slope	Intercept	R^2	RSD (%)
Pistachio	ALPLDVIK	2.5	6170.3	10029.0	0.992	21.7	11996.0	21362.0	0.983	20.3	7568.0	19876.3	0.991	45.0	18297.9	20827.9	0.973	13.5
	AMISPLAGSTSVLR	2.5	3429.9	8863.4	0.986	13.6	4346.7	4762.3	0.997	16.2	4282.5	4727.6	0.991	48.3	9349.0	7929.0	0.990	6.2
	LQELYETASELPR	2.5	1659.5	6547.4	0.971	13.1	1554.6	2188.0	0.995	12.6	2202.3	−1666.1	0.992	18.4	5590.0	11874.6	0.994	2.3
	VTSINALNLPILR	2.5	4695.9	17862.3	0.978	10.6	2280.9	−534.1	1.000	13.1	3011.3	11620.7	0.980	28.0	10201.7	15295.0	0.993	14.7
	ITSLNSLNLPILK	2.5	5415.7	20985.1	0.970	14.3	2753.2	462.8	1.000	12.4	5475.6	8025.2	0.994	41.4	12181.9	2710.6	0.994	7.5
Cashew	CAGVALVR	2.5	3846.5	8887.4	0.994	11.4	20936.5	11480.3	0.999	15.0	20078.6	−36837.1	0.994	10.7	4981.5	11751.8	0.994	20.7
	AMTSPLAGR	2.5	3695.6	10934.4	0.988	8.2	11610.3	2913.2	1.000	4.6	19597.6	−21358.5	0.991	10.6	17463.6	24538.8	0.995	18.4
	ADIYTPEVGR	2.5	7959.9	23539.4	0.984	7.9	13643.0	23274.2	0.999	7.0	23420.4	45312.2	0.992	9.8	23815.1	112750.3	0.988	18.3
	ELYETASELPR	2.5	3293.6	2771.3	0.999	10.9	7353.5	−2084.9	1.000	9.1	7964.8	−11087.2	0.993	8.5	13004.7	28424.7	0.991	5.3
Almond	YNRQETIALSSSQQR	25	107.5	139.0	–	28.1	10092.2	6308.3	–	8.0	7435.0	−3316.8	–	13.6	1011.8	−249.5	–	20.6
	QETIALSSSQQR	25	176.2	551.4	–	15.9	3378.4	3982.6	–	4.6	3147.9	5786.4	–	15.2	6410.8	9844.6	–	7.4
	ALPDEVLANAYQISR	5	197.0	1563.3	0.900	19.8	4856.9	5135.5	0.995	17.2	2848.5	561.3	0.993	21.5	278.6	269.5	0.977	8.7
	GNLDFVQPPR	5	535.6	3005.9	0.946	26.2	9935.7	1196.4	1.000	25.6	14317.0	23342.2	0.988	17.2	17658.9	40166.1	0.988	6.5
	TDENGFTNTLAGR	25	92.6	410.4	–	14.8	1389.9	−36.9	–	1.8	1714.9	1728.1	–	12.4	2029.7	182.7	–	32.6
Hazelnut	LNALEPTNR	5	497.2	−109.5	0.998	18.0	3341.8	5029.2	0.988	7.6	4731.3	14540.9	0.975	14.0	14296.7	−3685.4	0.999	5.8
	ADIYTEQVGR	5	997.9	736.8	0.992	13.7	8844.5	6355.8	0.999	4.2	8133.5	−6796.5	0.997	11.4	16895.3	12887.3	0.993	4.3
	TNDNAQISPLAGR	12.5	560.7	−126.8	0.999	11.5	8545.5	5607.3	1.000	9.7	7940.2	−9394.5	0.996	13.7	6045.4	1834.8	1.000	5.9
	INTVNSNTLPVLR	5	1586.4	−36.0	0.998	18.8	8503.5	8497.4	0.991	13.3	10787.4	−5098.2	0.998	12.3	16216.8	16445.0	0.998	15.8
	QGQVLTIPQNFAVAK	5	256.6	−87.0	0.998	12.2	1594.0	2659.1	0.992	12.9	1255.1	100.0	0.994	20.6	1144.2	483.3	0.996	15.1
	ALPDDVLANAFQISR	2.5	1442.2	673.0	0.997	11.4	3446.7	2111.1	0.999	13.0	3435.9	−787.2	0.994	11.6	2158.7	487.8	0.991	13.5
Pecan	QVESYFVPMER	25	835.8	−7106.9	0.952	21.0	2575.6	3121.7	0.996	13.0	1949.8	−10518.0	0.970	5.7	2288.0	2729.3	0.997	15.2
	ATLTFVSQER	12.5	849.3	−7091.8	0.978	19.1	1347.5	4481.1	0.998	10.0	1142.4	−3310.9	0.992	6.5	2017.0	8942.9	0.985	17.7
	NFLAGQNNIINQLER	12.5	643.3	−4046.5	0.967	16.2	1424.8	1890.1	0.998	6.3	2054.4	−5870.1	0.999	13.2	2415.0	6704.2	0.989	10.9
	LVGFGGINGK	5	2685.0	−5753.6	0.994	15.2	9344.9	−7730.7	0.991	40.5	7871.5	−3930.1	0.967	16.8	15155.9	4752.4	0.993	17.0
Pecan and walnut	LLQPVNNPGQFR	2.5	1503.3	−1425.6	0.998	16.6	7465.4	1325.1	1.000	14.5	7782.7	7642.4	0.996	15.8	10775.5	9352.6	0.992	15.4
	VFSNDILVAALNTPR	5	2664.1	−834.9	1.000	11.6	3378.7	4067.2	0.999	12.6	4072.6	256.7	0.991	10.5	5918.2	3507.4	0.994	13.9
Walnut	ATLTLVSQETR	10	1205.5	−6231.5	0.991	13.1	1258.3	4721.6	0.999	6.4	1673.8	−1.8	0.997	8.6	1876.2	1757.7	0.994	14.1
	GEEMEEMVQSAR	5	660.1	1033.5	0.991	13.3	888.6	715.7	0.996	9.1	837.6	4759.7	0.956	9.8	6001.9	11857.7	0.987	5.1
Milk	FFVAPFPEVFGK	0.5	25463.4	−546.6	0.990	30.9	7676.7	1937.5	0.988	8.4	32394.0	9297.6	0.991	4.3	68045.8	51763.2	0.989	5.1
	YLGYLEQLLR	0.5	4308.8	331.7	0.995	18.7	1023.4	465.9	0.994	15.5	1647.6	840.0	0.985	4.5	4542.9	3439.4	0.987	4.1
	HQGLPQEVLNENLLR	5	6512.2	9325.5	–	23.7	1602.2	−213.0	–	9.5	1943.1	263.7	–	6.1	7167.3	−2067.9	–	22.4
	NAVPITPTLNR	2.5	8940.3	3414.0	0.993	11.2	2390.9	1725.1	0.990	19.9	3163.7	6943.3	0.848	18.5	21031.0	8814.8	0.992	15.7
	VLVLVTDYK	10	1350.3	373.8	–	5.7	174.7	174.5	–	17.2	960.8	635.5	–	9.4	3277.4	1767.9	–	4.3
	VYVEELKPTPEGDLEILLQK	th	2180.2	−160.6	–	17.5	–	–	–	–	337.9	1095.7	–	10.4	1531.3	449.3	–	3.4
	LSFNPTQLEEQCHI	5	5803.8	788.9	–	8.2	331.9	123.1	–	7.1	2481.5	2461.3	–	10.5	4967.6	1523.7	–	4.3
Egg	GGLEPINFQTAADQAR	3	1317.7	2897.6	0.995	11.3	1185.2	2769.6	0.997	10.5	8973.3	14539.2	0.987	4.5	12403.4	37409.1	0.994	3.6
	ISQAVHAAHAEINEAGR	3	38.9	125.7	0.975	25.1	108.6	582.9	0.986	18.7	388.4	2614.6	0.888	6.9	226.8	12.9	0.981	6.0
	LTEWTSSNVMEER	15	446.1	848.3	0.995	8.8	262.8	647.9	0.986	18.0	1663.9	12462.7	0.923	10.5	3401.2	7427.0	0.997	13.4
	YLLDLLPAAASHR	60	434.3	767.9	–	11.5	18.3	102.1	–	16.5	212.6	1795.9	–	9.1	244.0	1201.4	–	6.0
	EALQPIHDLADEAISR	60	1632.5	5310.0	–	10.8	75.2	−84.8	–	11.2	1065.7	5763.6	–	9.5	1222.9	1433.1	–	4.6
	NIPFAEYPTYK	60	1689.8	1807.3	–	8.9	138.1	−568.2	–	13.7	861.8	−610.7	–	9.8	1604.9	5867.5	–	8.4
Peanut	TANELNLLILR	2.5	1327.8	903.8	1.000	8.3	730.6	2007.7	0.995	15.3	905.2	2974.3	0.976	9.9	2276.5	10575.1	0.989	2.5
	RPYSNAPQEIFIQQGR	5	–	–	–	–	2586.7	7689.0	0.995	11.1	2234.9	1288.0	0.986	9.9	1323.9	1045.0	0.944	17.6
	FNLAGNHEQEFLR	50	109.9	−256.2	–	19.9	460.4	1354.0	–	16.5	452.3	4027.3	–	13.8	1785.3	93.1	–	7.9
	NLPQQCGLR	5	1434.1	−125.8	0.998	9.3	2545.1	10184.2	0.978	7.8	909.0	4852.7	0.935	36.7	2566.4	11133.0	0.973	3.8
Soy	EAFGVNMQIVR	5	1890.9	2858.5	1.000	6.2	1142.1	7116.1	0.997	6.7	1309.2	1663.8	0.986	7.0	2114.7	11837.9	0.996	5.3
	ELINLATMCR	5	1818.1	−1513.1	0.999	9.4	882.9	7072.3	0.990	9.9	490.9	1238.0	0.988	6.3	1335.4	10159.4	0.978	6.6
	LITAIPVNKPGR	25	2210.5	−4339.2	0.998	20.2	386.3	2815.0	0.970	26.3	705.5	5096.1	0.954	17.4	5364.4	23272.7	0.992	6.0
	VFDGELQEGR	10	781.8	3262.0	0.996	10.0	482.0	4409.7	0.988	8.2	751.4	7823.0	0.927	8.4	812.8	5500.3	0.992	3.7
	ISTNLNSLTPALR	10	1348.0	10320.5	0.986	5.0	101.8	−282.1	0.961	16.0	197.6	2952.4	0.799	8.6	663.2	5450.5	0.977	5.8

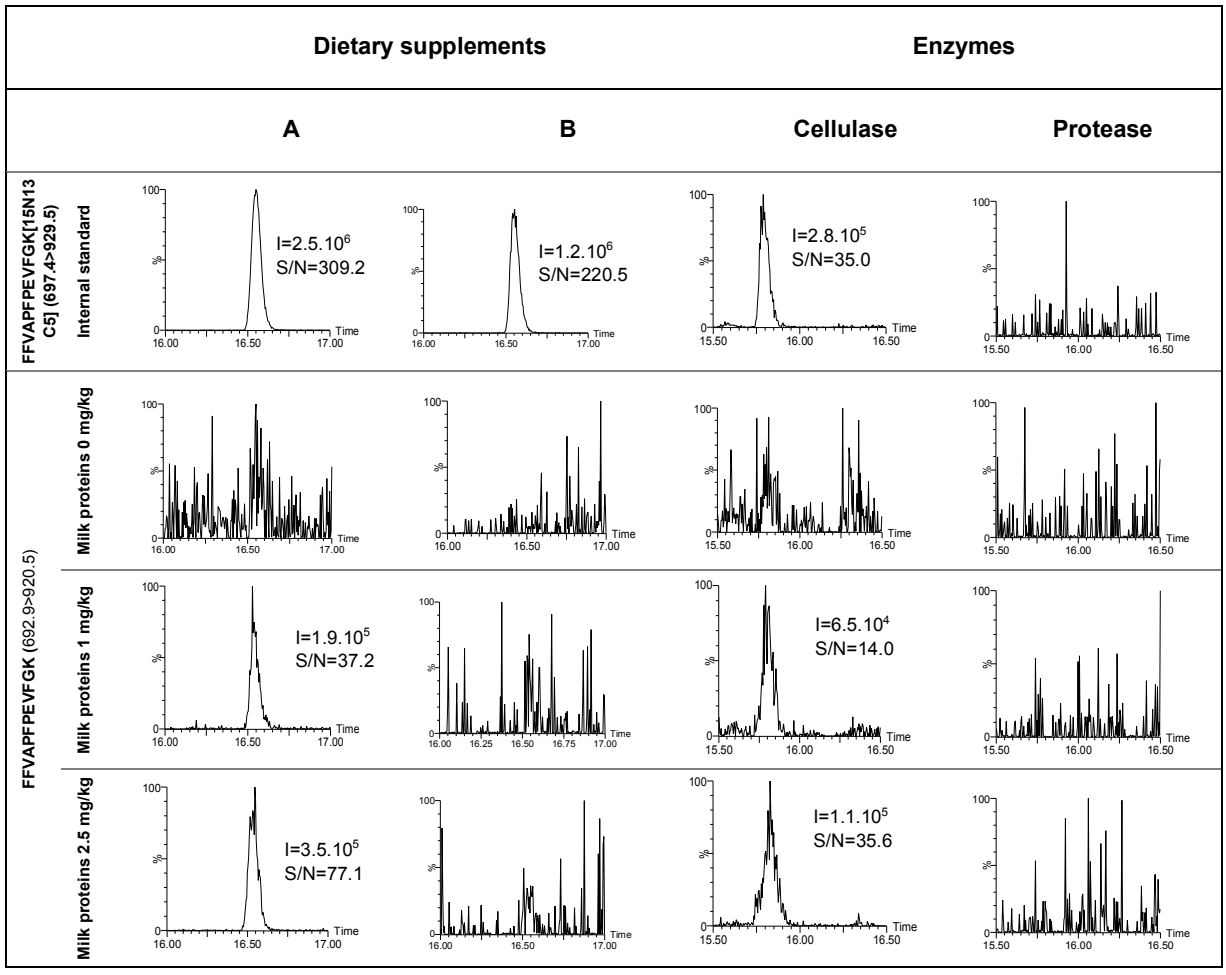

Fig. 2. Chromatograms of the highest multiple reaction monitoring MRM transtions of milk casein peptide FFVAPFPEVGK (692.9 > 920.5) in real samples spiked at 0, 1, and 2.5 mg milk proteins per kg. Each sample was spiked at 13 ng/mL with labeled casein peptide FFVAPFPEVFGK [$^{13}C_6{}^{15}N_2$].

tion of trypsin activity. Use of an isotope-labeled peptide cannot show whether trypsin is inhibited or not. A way to monitor the efficiency of the enzymatic digestion could be to use labeled proteins or long isotope-labeled peptides (concatamers). To ensure the detection of milk in samples with a high trypsin inhibitor protein content, another enzymatic digestion strategy (other enzymes, denaturing conditions) could be tested. In the sample containing cellulase, the method was able to detect milk allergen correctly after standard addition at 1 mg and 2.5 mg milk proteins per kg. In the case of the protease samples, the use of labeled peptides revealed either a major matrix effect or, more probably, the hydrolysis of proteins and labeled peptide, making it impossible to detect specific peptides. In routine laboratories, criteria for determining a negative result are based on the absence of an MRM signal and detection of the internal standard. Ideally, the isotope-labeled standard must be able to correct for both the matrix effect and enzymatic digestion efficiency to guarantee the absence of false negative results. Introducing long isotope-labeled peptides, requiring digestion, should probably reveal the absence of enzyme digestion and avoid false negative results.

4. Conclusion

The goal of this study was to develop a suitable method for the analysis of 10 allergens, applicable in one day with a single protocol. The rapidity of the method described here and its capacity to detect 10 allergens in complex and incurred food products with high specificity and sensitivity make it suitable for use in routine laboratories. Our multi-allergen detection method has the lowest limits of quantification available to date for incurred foodstuffs: 0.5 mg milk proteins/kg, 2.5 mg peanut, cashew, hazelnut, and pistachio proteins/kg, 3.0 mg egg proteins/kg, and 5 mg soybean, walnut, pecan, and almond proteins/kg, applicable to four different matrices. The method also shows an LOD (S/N > 3) below the VITAL threshold (portion size: 40 g) for all the target allergens except yolk egg (60 mg egg proteins per kg) and milk whey proteins (5 mg milk proteins per kg), which can easily be separated, respectively, from egg white and caseins in food preparations. The developed method was able to detect two positive complex samples out of four, while the matrix effect was too high for use of the ELISA method. In the present study, 90.7% of the coefficients of regression were higher than 0.97 and only 11.6% of the RSDs were higher than 20%. Furthermore, correction by means of a labeled internal standard reduced the RSD for milk casein peptide from 30.9% to 10.3%. Our analyses of real and incurred samples have shown the importance of introducing suitable labeled standards in order to correct for matrix effects and to check the capacity of the method to detect target allergens. With a view to developing a routine quantitative method, we plan to develop a single-run injection including the 10 target allergens and considering only three peptides per allergen. Long isotope-labeled standards will be tested for corrections linked to matrix effects and protocol step efficiency. In this manner, we expect to achieve better recoveries for allergen quantification than with labeled peptides.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.chroma.2017.11.039.

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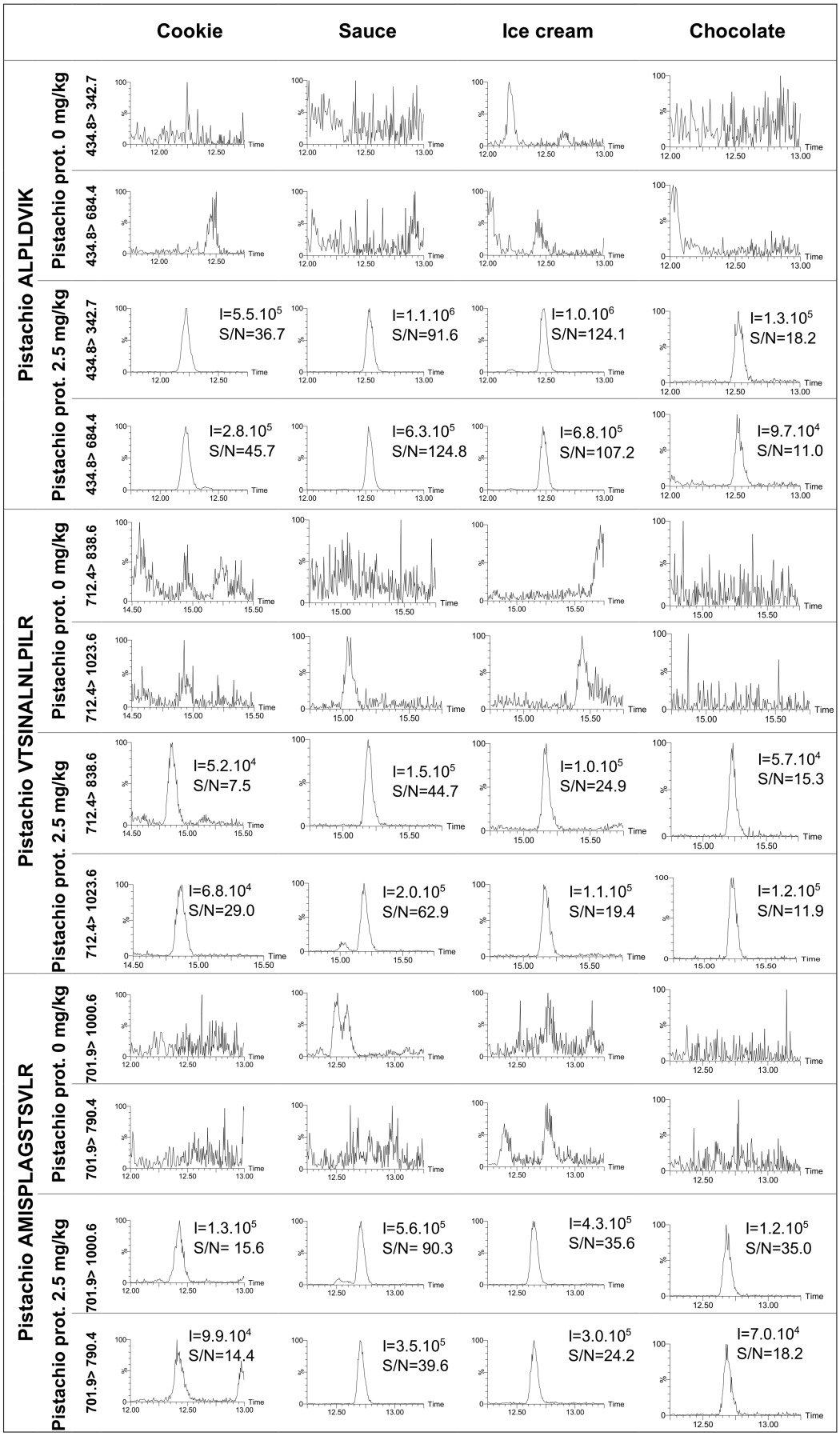
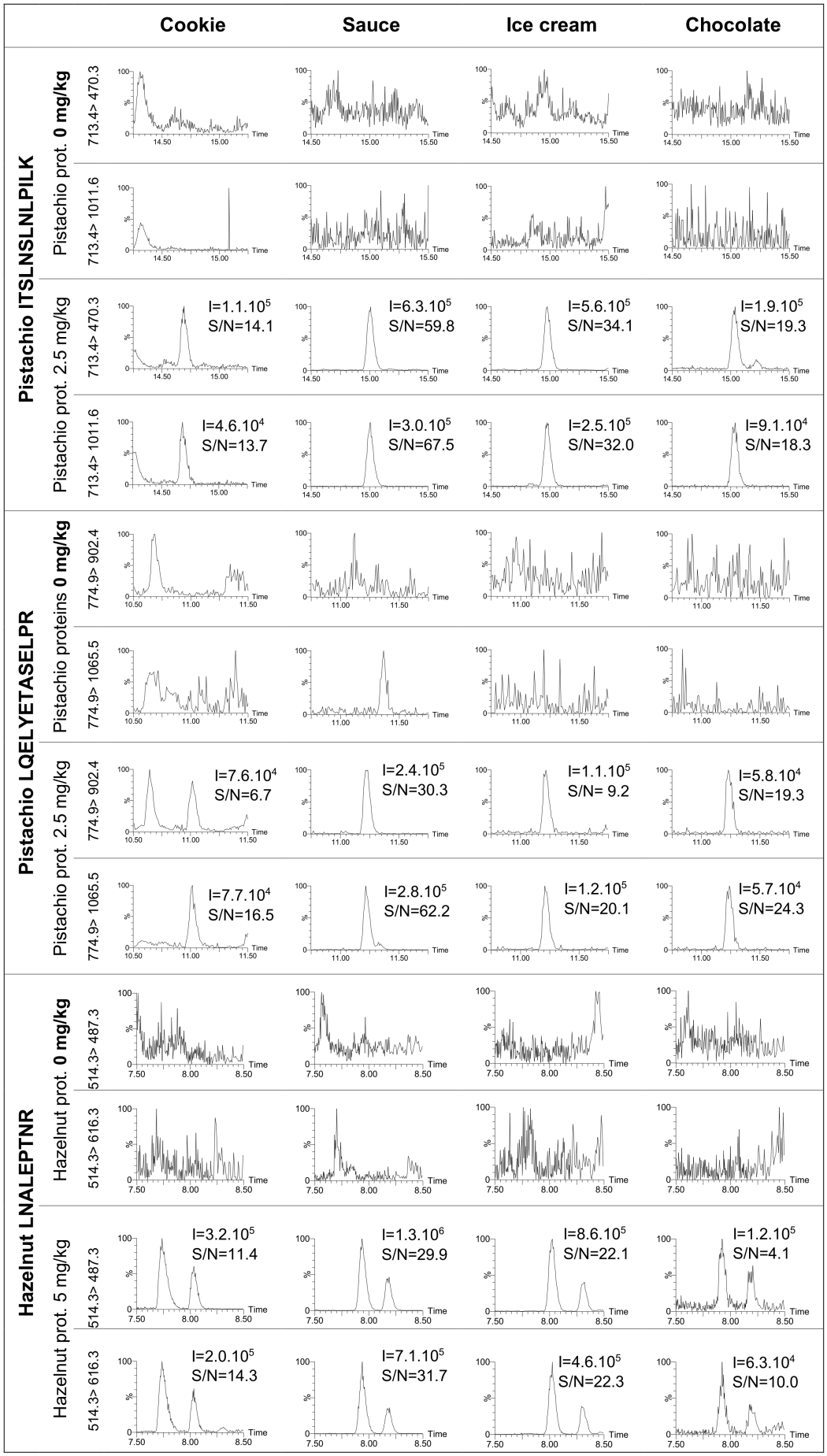
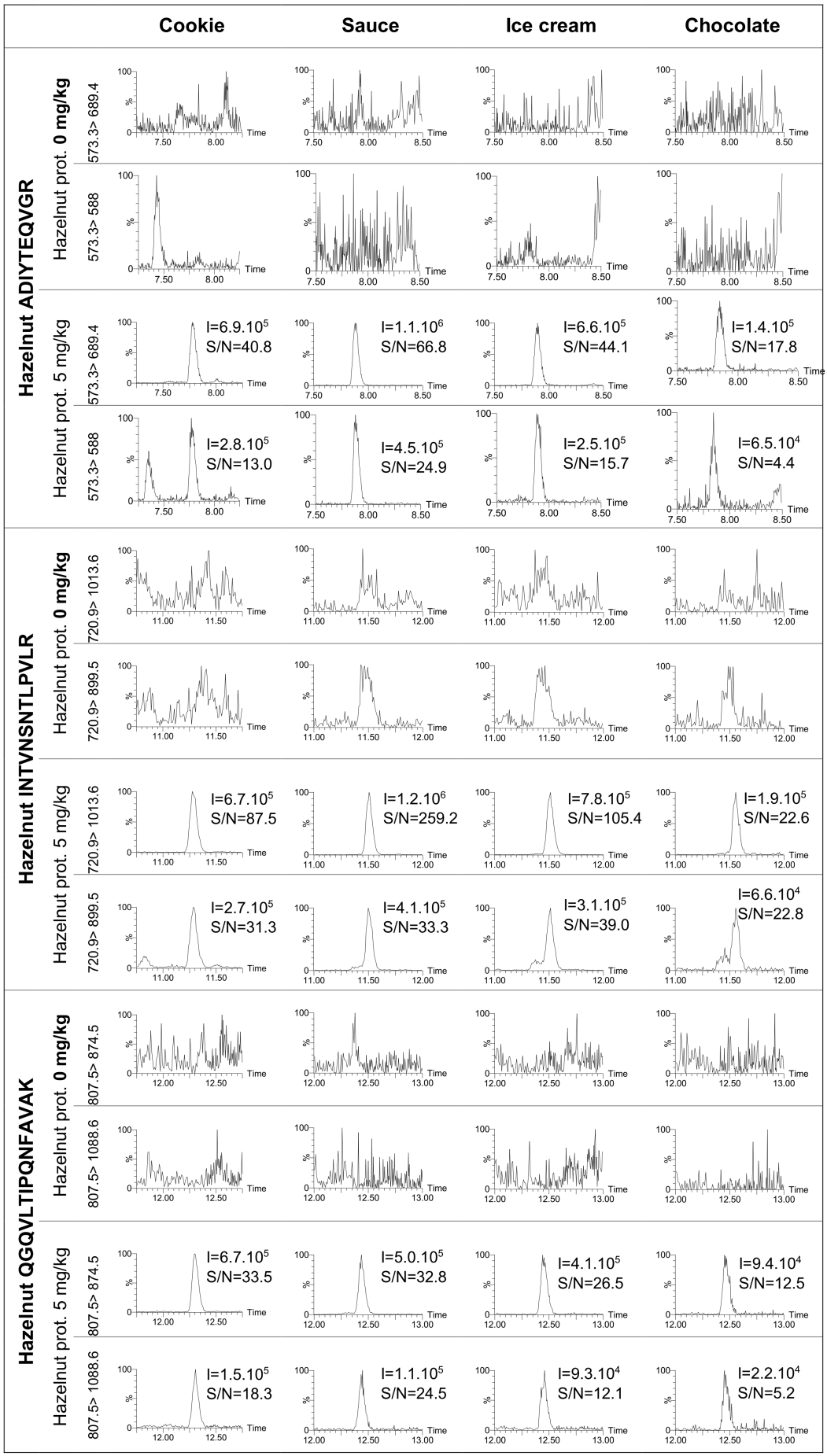
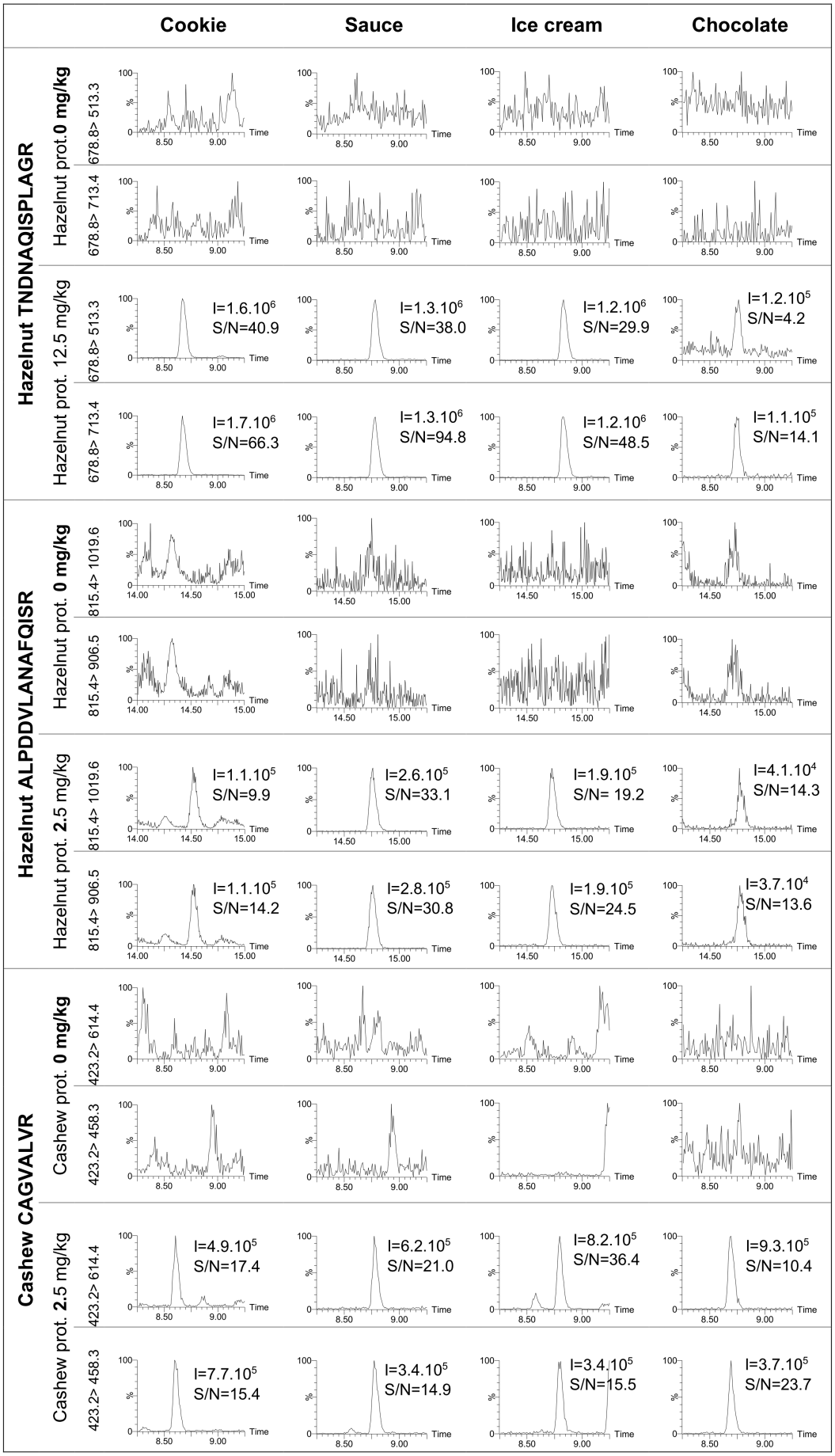
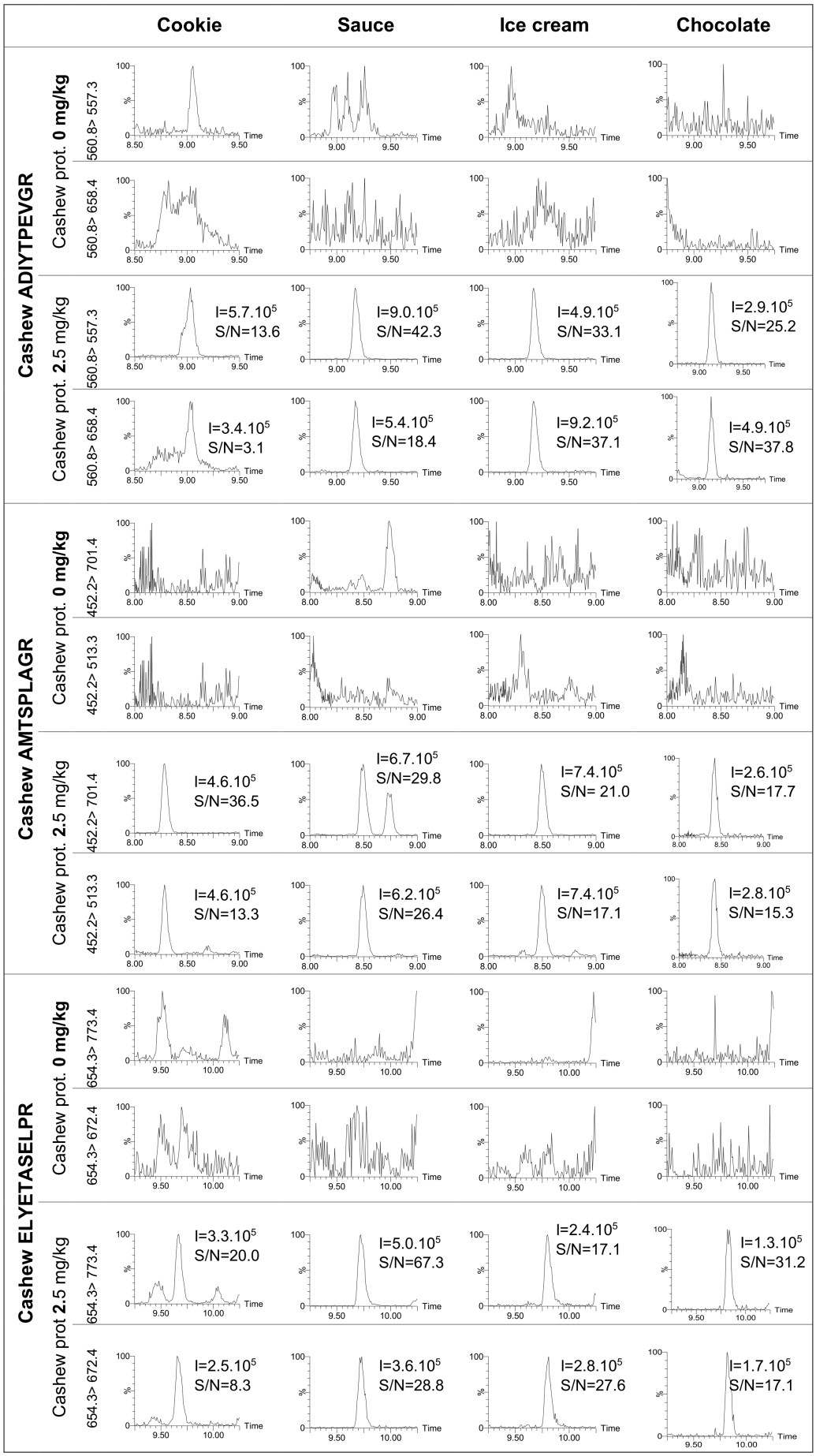
Complementary figure 1: Chromatograms of the two highest multiple reaction monitoring (MRM) transitions of milk, egg, soy, peanut, pistachio, hazelnut, cashew, almond, walnut, and pecan nut allergens. Data for non-contaminated matrices (0 mg/kg) and for incurred matrices at the limit of quantification are presented without smoothing (complementary data).


Cookie
Sauce
Ice cream
Chocolate
Pistachio ALPLDVIK
Pistachio prot. 0 mg/kg
434.8> 342.7
434.8> 684.4
Pistachio prot. 2.5 mg/kg
434.8> 342.7
I=5.5.10^5 S/N=36.7
I=1.1.10^6 S/N=91.6
I=1.0.10^6 S/N=124.1
I=1.3.10^5 S/N=18.2
434.8> 684.4
I=2.8.10^5 S/N=45.7
I=6.3.10^5 S/N=124.8
I=6.8.10^5 S/N=107.2
I=9.7.10^4 S/N=11.0
Pistachio VTSINALNLPILR
Pistachio prot. 0 mg/kg
712.4> 838.6
712.4> 1023.6
Pistachio prot. 2.5 mg/kg
712.4> 838.6
I=5.2.10^4 S/N=7.5
I=1.5.10^5 S/N=44.7
I=1.0.10^5 S/N=24.9
I=5.7.10^4 S/N=15.3
712.4> 1023.6
I=6.8.10^4 S/N=29.0
I=2.0.10^5 S/N=62.9
I=1.1.10^5 S/N=19.4
I=1.2.10^5 S/N=11.9
Pistachio AMISPLAGSTSVLR
Pistachio prot. 0 mg/kg
701.9> 1000.6
701.9> 790.4
Pistachio prot. 2.5 mg/kg
701.9> 1000.6
I=1.3.10^5 S/N= 15.6
I=5.6.10^5 S/N= 90.3
I=4.3.10^5 S/N=35.6
I=1.2.10^5 S/N=35.0
701.9> 790.4
I=9.9.10^4 S/N=14.4
I=3.5.10^5 S/N=39.6
I=3.0.10^5 S/N=24.2
I=7.0.10^4 S/N=18.2


Cookie
Sauce
Ice cream
Chocolate
Pistachio ITSLNSLNLPILK
Pistachio prot. 0 mg/kg
713.4> 470.3
713.4> 1011.6
Pistachio prot. 2.5 mg/kg
713.4> 470.3
I=1.1.10^5 S/N=14.1
I=6.3.10^5 S/N=59.8
I=5.6.10^5 S/N=34.1
I=1.9.10^5 S/N=19.3
713.4> 1011.6
I=4.6.10^4 S/N=13.7
I=3.0.10^5 S/N=67.5
I=2.5.10^5 S/N=32.0
I=9.1.10^4 S/N=18.3
Pistachio LQELYETASELPR
Pistachio proteins 0 mg/kg
774.9> 902.4
774.9> 1065.5
Pistachio prot. 2.5 mg/kg
774.9> 902.4
I=7.6.10^4 S/N=6.7
I=2.4.10^5 S/N=30.3
I=1.1.10^5 S/N= 9.2
I=5.8.10^4 S/N=19.3
774.9> 1065.5
I=7.7.10^4 S/N=16.5
I=2.8.10^5 S/N=62.2
I=1.2.10^5 S/N=20.1
I=5.7.10^4 S/N=24.3
Hazelnut LNALEPTNR
Hazelnut prot. 0 mg/kg
514.3> 487.3
514.3> 616.3
Hazelnut prot. 5 mg/kg
514.3> 487.3
I=3.2.10^5 S/N=11.4
I=1.3.10^6 S/N=29.9
I=8.6.10^5 S/N=22.1
I=1.2.10^5 S/N=4.1
514.3> 616.3
I=2.0.10^5 S/N=14.3
I=7.1.10^5 S/N=31.7
I=4.6.10^5 S/N=22.3
I=6.3.10^4 S/N=10.0
Time


Cookie
Sauce
Ice cream
Chocolate
Hazelnut ADIYTEQVGR
Hazelnut prot. 0 mg/kg
573.3> 689.4
573.3> 588
Hazelnut prot. 5 mg/kg
573.3> 689.4
I=6.9.10^5 S/N=40.8
I=1.1.10^6 S/N=66.8
I=6.6.10^5 S/N=44.1
I=1.4.10^5 S/N=17.8
573.3> 588
I=2.8.10^5 S/N=13.0
I=4.5.10^5 S/N=24.9
I=2.5.10^5 S/N=15.7
I=6.5.10^4 S/N=4.4
Hazelnut INTVNSNTLPVLR
Hazelnut prot. 0 mg/kg
720.9> 1013.6
720.9> 899.5
Hazelnut prot. 5 mg/kg
720.9> 1013.6
I=6.7.10^5 S/N=87.5
I=1.2.10^6 S/N=259.2
I=7.8.10^5 S/N=105.4
I=1.9.10^5 S/N=22.6
720.9> 899.5
I=2.7.10^5 S/N=31.3
I=4.1.10^5 S/N=33.3
I=3.1.10^5 S/N=39.0
I=6.6.10^4 S/N=22.8
Hazelnut QGQVLTIPQNFAVAK
Hazelnut prot. 0 mg/kg
807.5> 874.5
807.5> 1088.6
Hazelnut prot. 5 mg/kg
807.5> 874.5
I=6.7.10^5 S/N=33.5
I=5.0.10^5 S/N=32.8
I=4.1.10^5 S/N=26.5
I=9.4.10^4 S/N=12.5
807.5> 1088.6
I=1.5.10^5 S/N=18.3
I=1.1.10^5 S/N=24.5
I=9.3.10^4 S/N=12.1
I=2.2.10^4 S/N=5.2


Cookie
Sauce
Ice cream
Chocolate
Hazelnut TNDNAQISPLAGR
Hazelnut prot.0 mg/kg
678.8> 513.3
678.8> 713.4
Hazelnut prot. 12.5 mg/kg
678.8> 513.3
I=1.6.10^6 S/N=40.9
I=1.3.10^6 S/N=38.0
I=1.2.10^6 S/N=29.9
I=1.2.10^5 S/N=4.2
678.8> 713.4
I=1.7.10^6 S/N=66.3
I=1.3.10^6 S/N=94.8
I=1.2.10^6 S/N=48.5
I=1.1.10^5 S/N=14.1
Hazelnut ALPDDVLANAFQISR
Hazelnut prot. 0 mg/kg
815.4> 1019.6
815.4> 906.5
Hazelnut prot. 2.5 mg/kg
815.4> 1019.6
I=1.1.10^5 S/N=9.9
I=2.6.10^5 S/N=33.1
I=1.9.10^5 S/N= 19.2
I=4.1.10^4 S/N=14.3
815.4> 906.5
I=1.1.10^5 S/N=14.2
I=2.8.10^5 S/N=30.8
I=1.9.10^5 S/N=24.5
I=3.7.10^4 S/N=13.6
Cashew CAGVALVR
Cashew prot. 0 mg/kg
423.2> 614.4
423.2> 458.3
Cashew prot. 2.5 mg/kg
423.2> 614.4
I=4.9.10^5 S/N=17.4
I=6.2.10^5 S/N=21.0
I=8.2.10^5 S/N=36.4
I=9.3.10^5 S/N=10.4
423.2> 458.3
I=7.7.10^5 S/N=15.4
I=3.4.10^5 S/N=14.9
I=3.4.10^5 S/N=15.5
I=3.7.10^5 S/N=23.7


Cookie
Sauce
Ice cream
Chocolate
Cashew ADIYTPEVGR
Cashew prot. 0 mg/kg
560.8> 557.3
560.8> 658.4
Cashew prot. 2.5 mg/kg
560.8> 557.3
I=5.7.10^5 S/N=13.6
I=9.0.10^5 S/N=42.3
I=4.9.10^5 S/N=33.1
I=2.9.10^5 S/N=25.2
560.8> 658.4
I=3.4.10^5 S/N=3.1
I=5.4.10^5 S/N=18.4
I=9.2.10^5 S/N=37.1
I=4.9.10^5 S/N=37.8
Cashew AMTSPLAGR
Cashew prot. 0 mg/kg
452.2> 701.4
452.2> 513.3
Cashew prot. 2.5 mg/kg
452.2> 701.4
I=4.6.10^5 S/N=36.5
I=6.7.10^5 S/N=29.8
I=7.4.10^5 S/N= 21.0
I=2.6.10^5 S/N=17.7
452.2> 513.3
I=4.6.10^5 S/N=13.3
I=6.2.10^5 S/N=26.4
I=7.4.10^5 S/N=17.1
I=2.8.10^5 S/N=15.3
Cashew ELYETASELPR
Cashew prot. 0 mg/kg
654.3> 773.4
654.3> 672.4
Cashew prot 2.5 mg/kg
654.3> 773.4
I=3.3.10^5 S/N=20.0
I=5.0.10^5 S/N=67.3
I=2.4.10^5 S/N=17.1
I=1.3.10^5 S/N=31.2
654.3> 672.4
I=2.5.10^5 S/N=8.3
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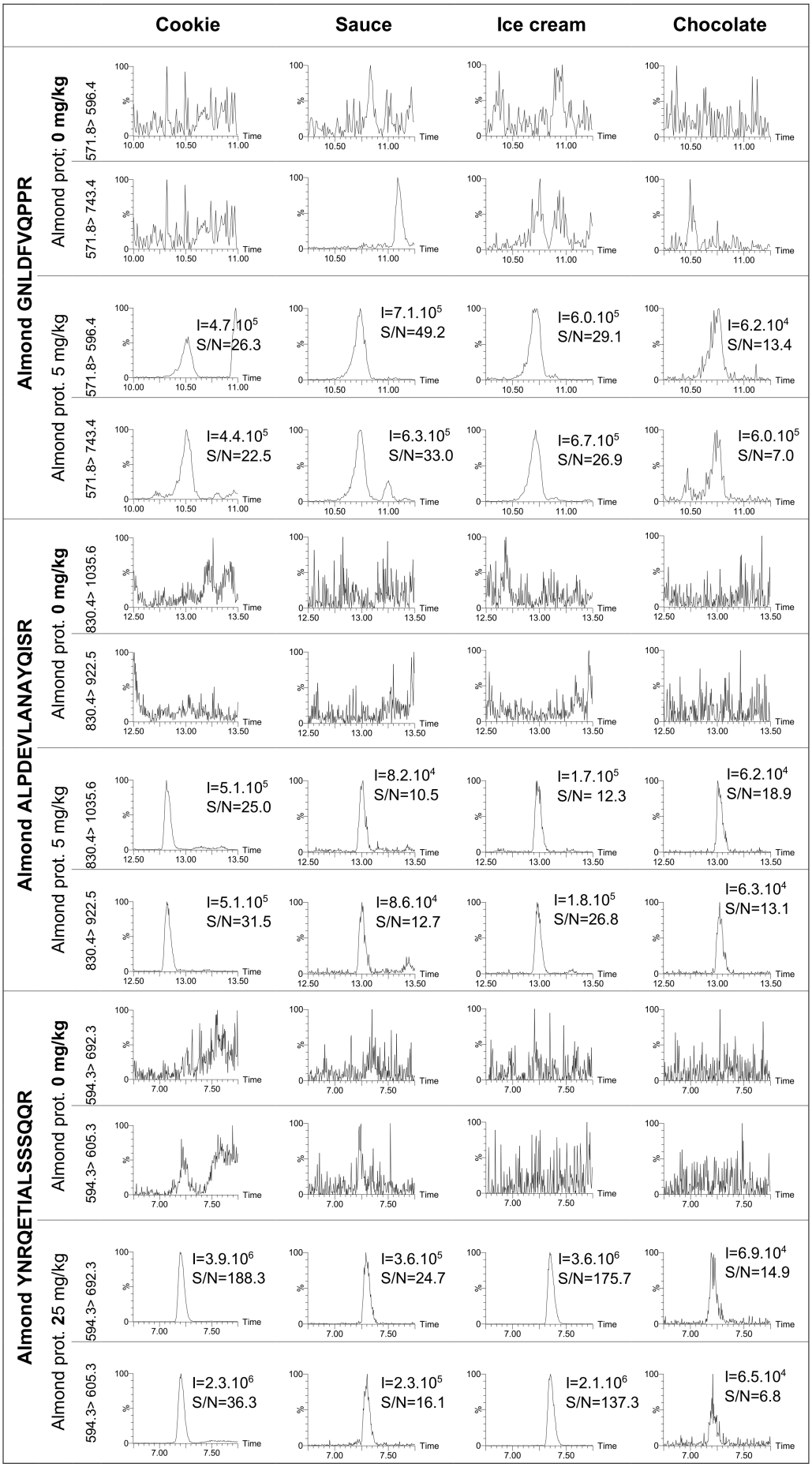
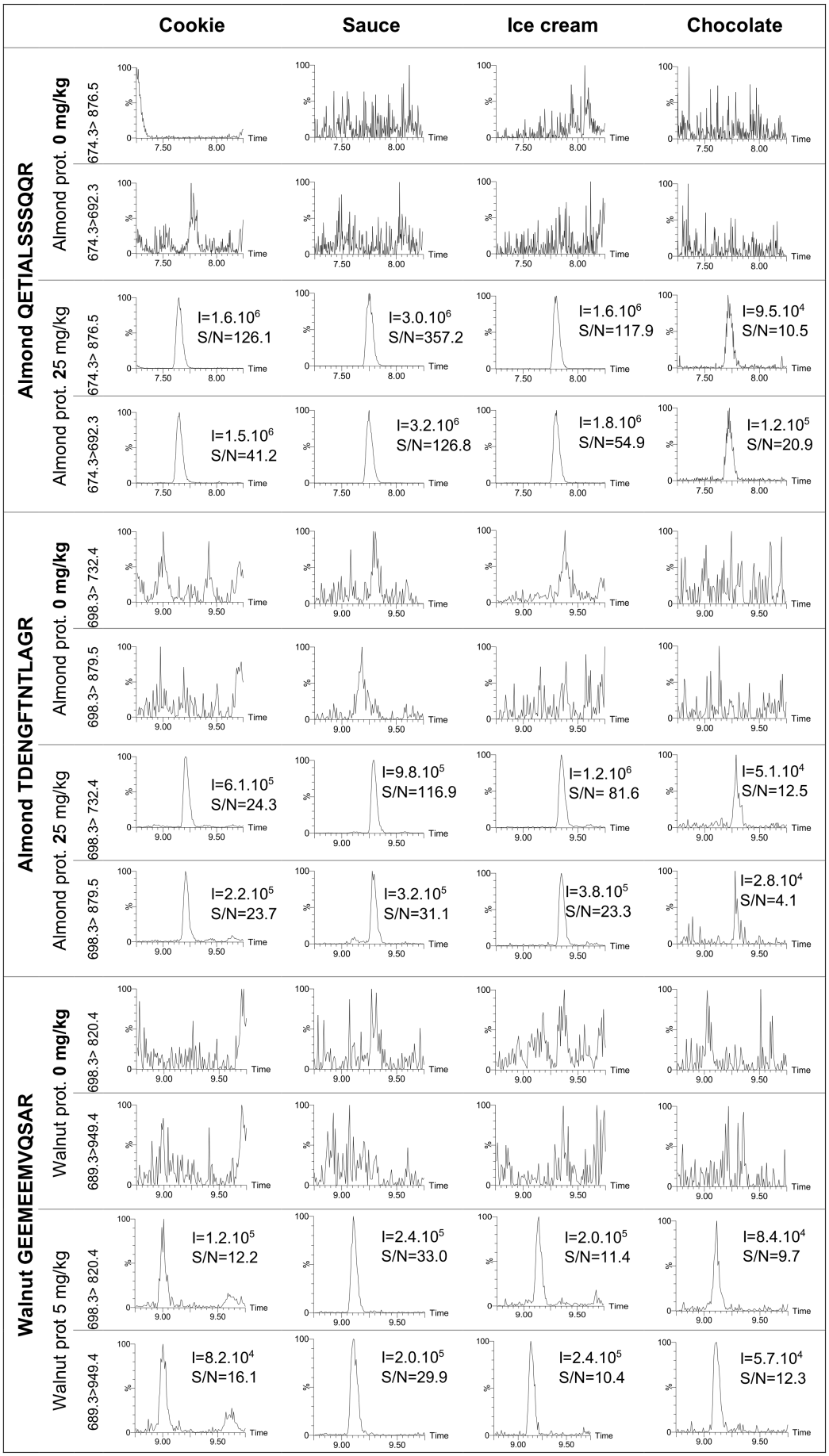
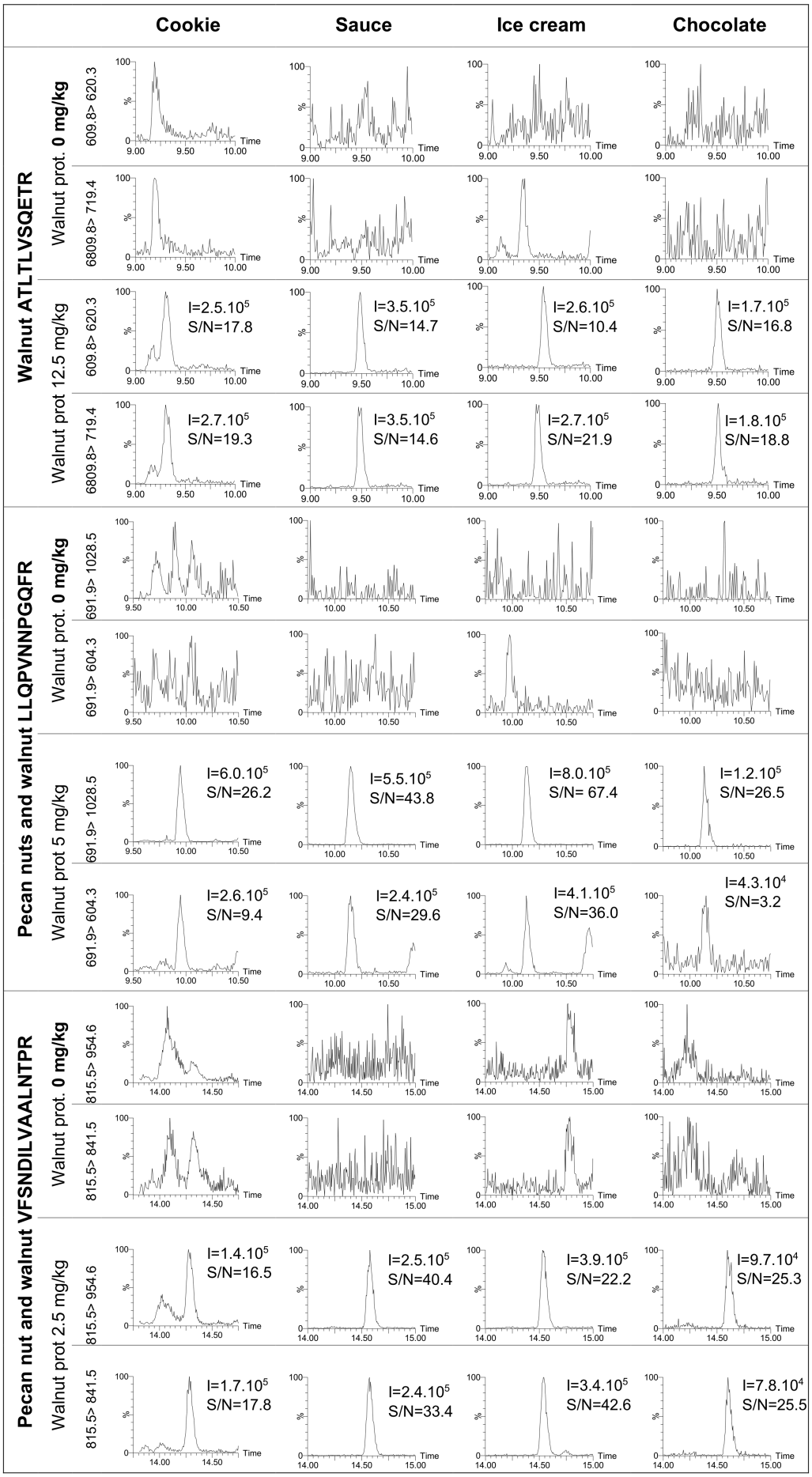
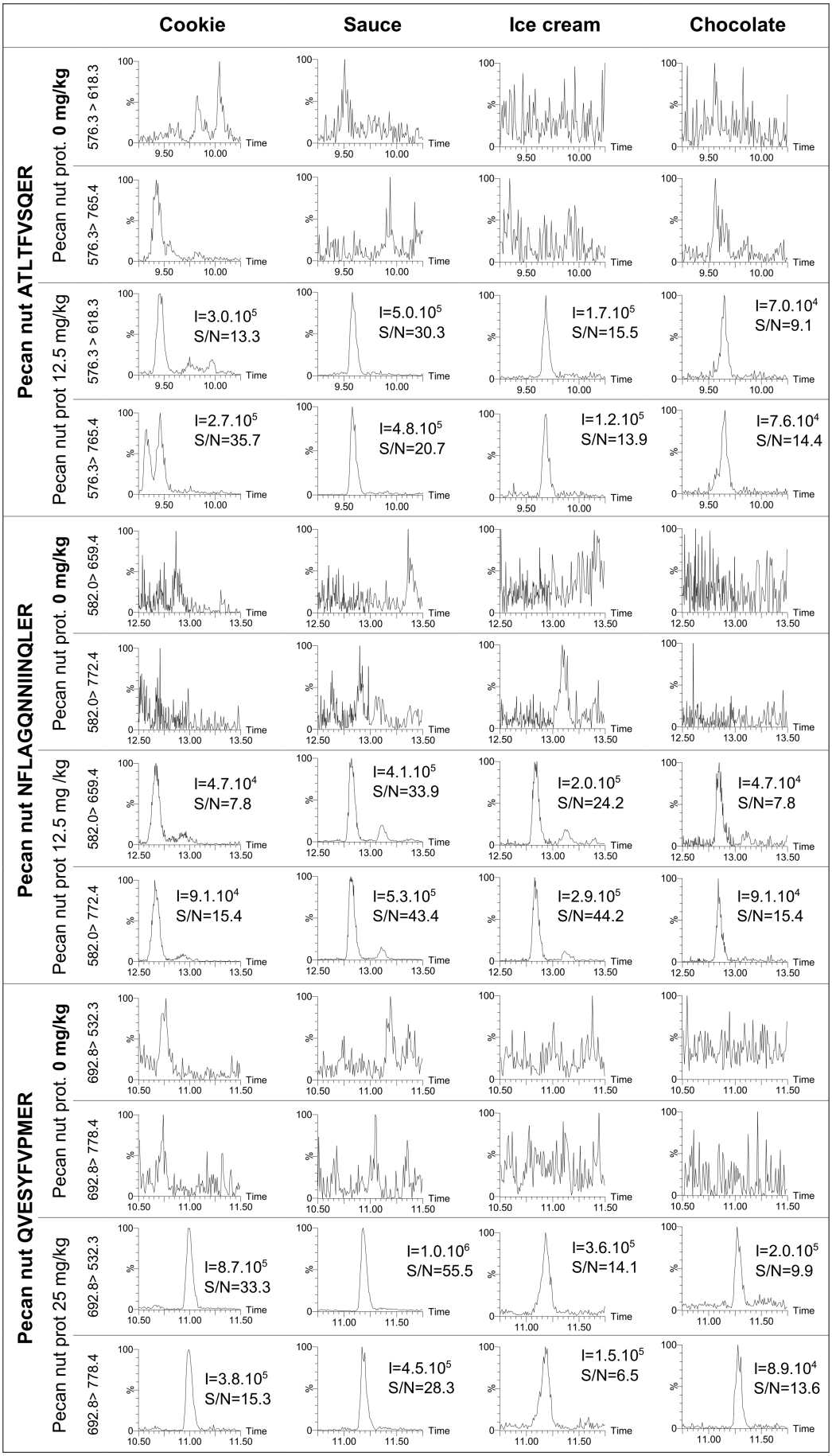
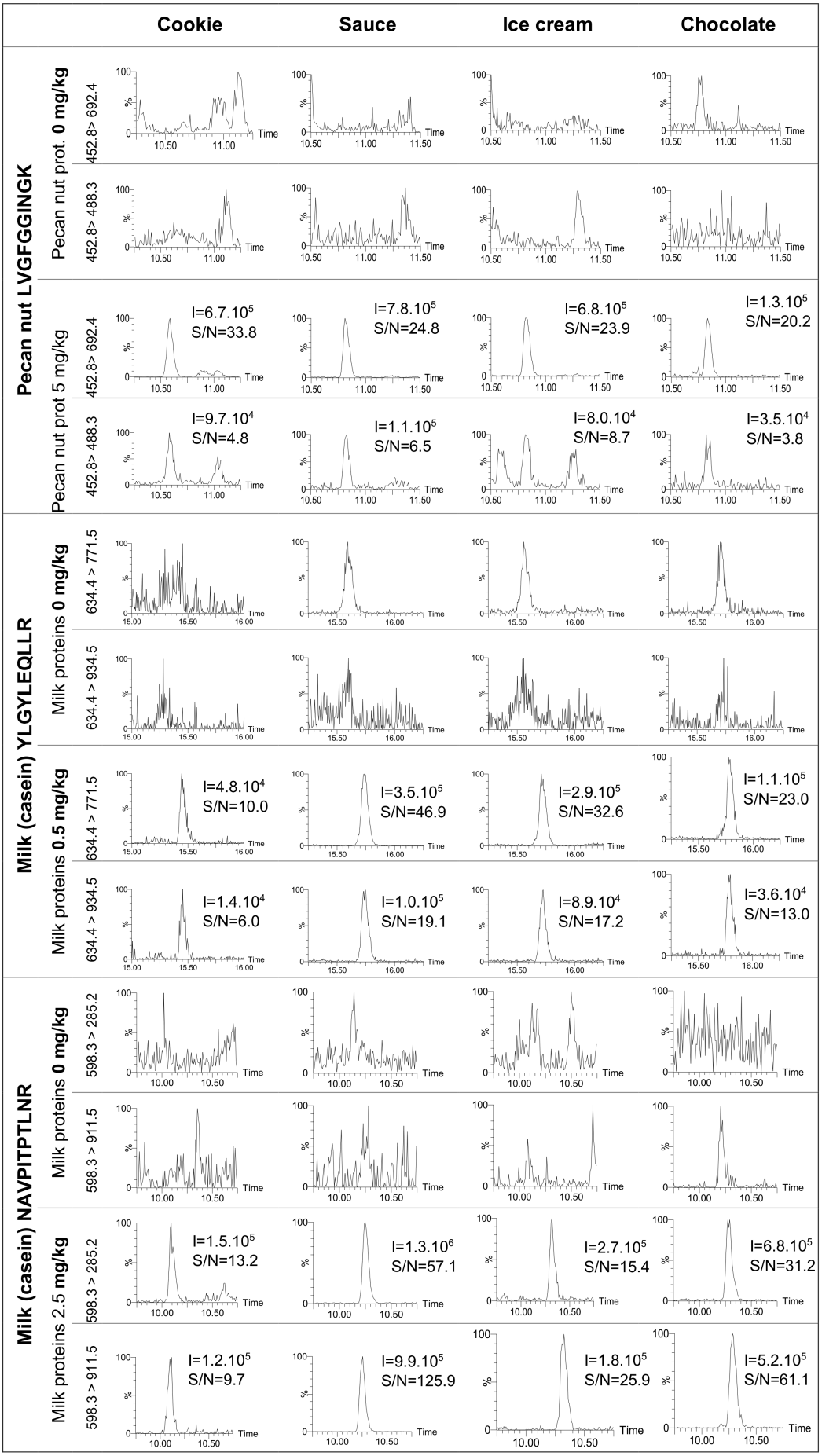
I=1.7.10^5 S/N=17.1

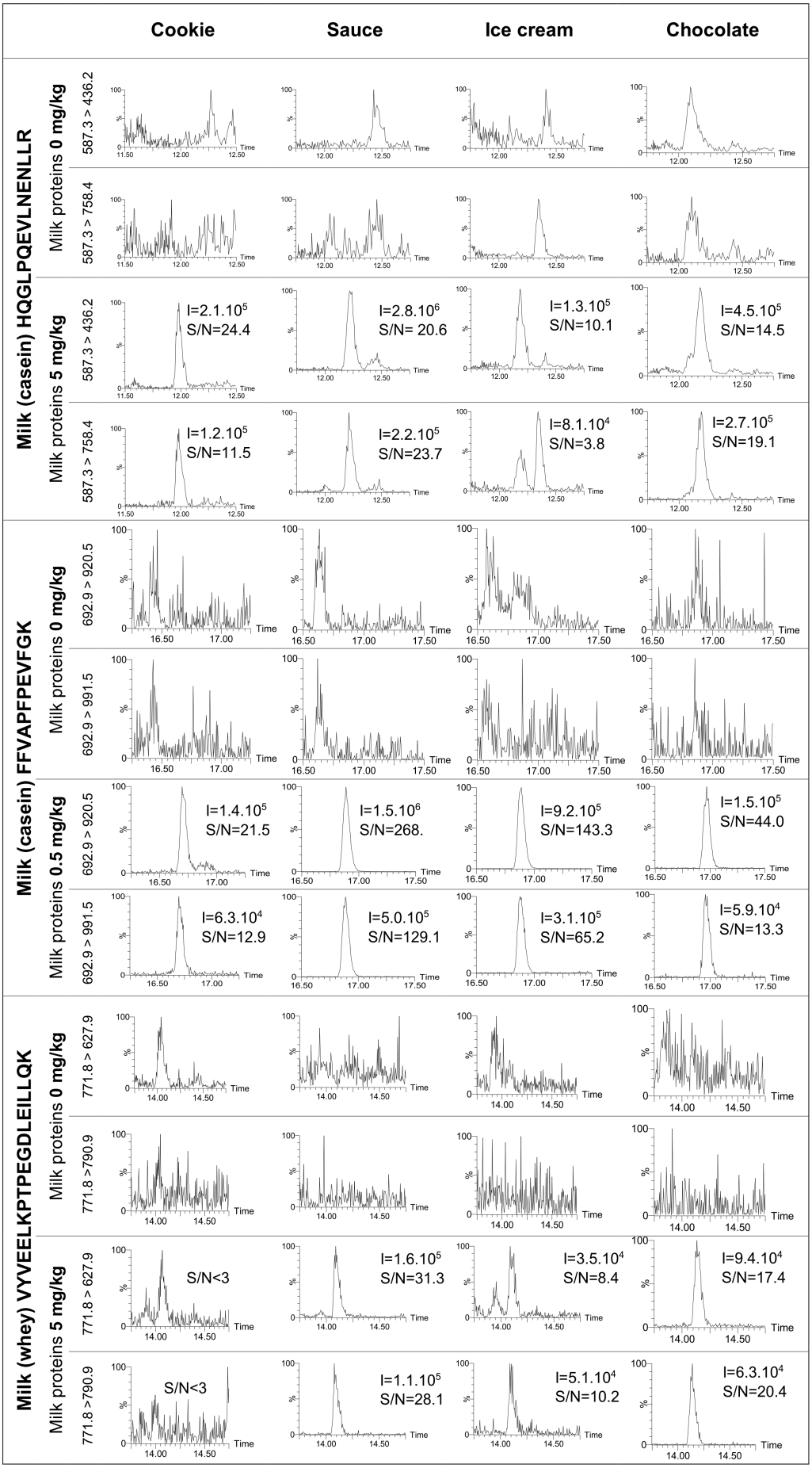

Cookie
Sauce
Ice cream
Chocolate
Almond GNLDFVQPPR
Almond prot; 0 mg/kg
571.8> 596.4
571.8> 743.4
Almond prot. 5 mg/kg
571.8> 596.4
I=4.7.10^5 S/N=26.3
I=7.1.10^5 S/N=49.2
I=6.0.10^5 S/N=29.1
I=6.2.10^4 S/N=13.4
571.8> 743.4
I=4.4.10^5 S/N=22.5
I=6.3.10^5 S/N=33.0
I=6.7.10^5 S/N=26.9
I=6.0.10^5 S/N=7.0
Almond ALPDEVLANAYQISR
Almond prot. 0 mg/kg
830.4> 1035.6
830.4> 922.5
Almond prot. 5 mg/kg
830.4> 1035.6
I=5.1.10^5 S/N=25.0
I=8.2.10^4 S/N=10.5
I=1.7.10^5 S/N= 12.3
I=6.2.10^4 S/N=18.9
830.4> 922.5
I=5.1.10^5 S/N=31.5
I=8.6.10^4 S/N=12.7
I=1.8.10^5 S/N=26.8
I=6.3.10^4 S/N=13.1
Almond YNRQETIALSSSQQR
Almond prot. 0 mg/kg
594.3> 692.3
594.3> 605.3
Almond prot. 25 mg/kg
594.3> 692.3
I=3.9.10^6 S/N=188.3
I=3.6.10^5 S/N=24.7
I=3.6.10^6 S/N=175.7
I=6.9.10^4 S/N=14.9
594.3> 605.3
I=2.3.10^6 S/N=36.3
I=2.3.10^5 S/N=16.1
I=2.1.10^6 S/N=137.3
I=6.5.10^4 S/N=6.8

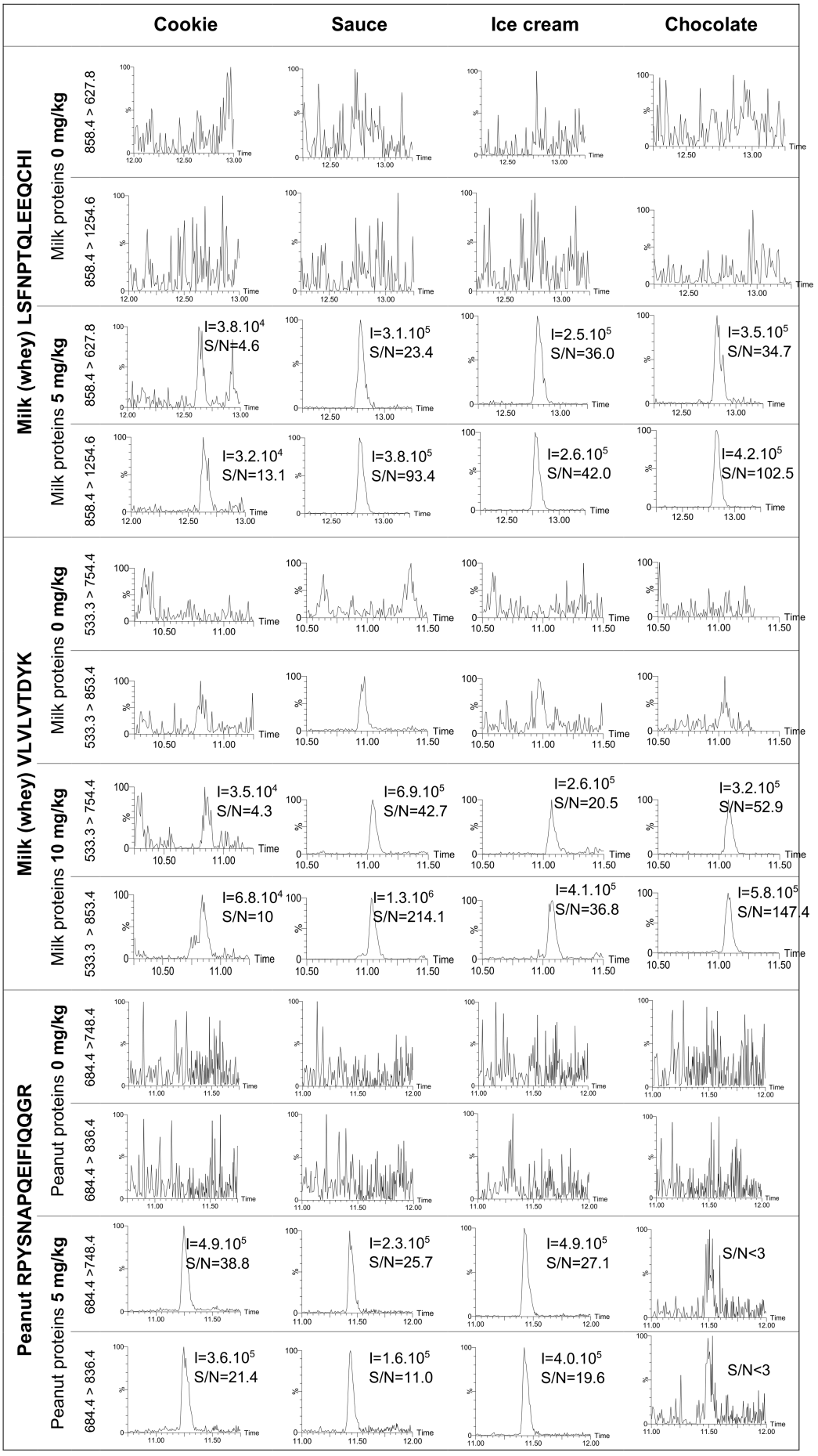

Cookie
Sauce
Ice cream
Chocolate
Almond QETIALSSSQQR
Almond prot. 0 mg/kg
674.3> 876.5
674.3>692.3
Almond prot. 25 mg/kg
674.3> 876.5
I=1.6.10^6 S/N=126.1
I=3.0.10^6 S/N=357.2
I=1.6.10^6 S/N=117.9
I=9.5.10^4 S/N=10.5
674.3>692.3
I=1.5.10^6 S/N=41.2
I=3.2.10^6 S/N=126.8
I=1.8.10^6 S/N=54.9
I=1.2.10^5 S/N=20.9
Almond TDENGFTNTLAGR
Almond prot. 0 mg/kg
698.3> 732.4
698.3> 879.5
Almond prot. 25 mg/kg
698.3> 732.4
I=6.1.10^5 S/N=24.3
I=9.8.10^5 S/N=116.9
I=1.2.10^6 S/N= 81.6
I=5.1.10^4 S/N=12.5
698.3> 879.5
I=2.2.10^5 S/N=23.7
I=3.2.10^5 S/N=31.1
I=3.8.10^5 S/N=23.3
I=2.8.10^4 S/N=4.1
Walnut GEEMEEMVQSAR
Walnut prot. 0 mg/kg
698.3> 820.4
689.3>949.4
Walnut prot 5 mg/kg
698.3> 820.4
I=1.2.10^5 S/N=12.2
I=2.4.10^5 S/N=33.0
I=2.0.10^5 S/N=11.4
I=8.4.10^4 S/N=9.7
689.3>949.4
I=8.2.10^4 S/N=16.1
I=2.0.10^5 S/N=29.9
I=2.4.10^5 S/N=10.4
I=5.7.10^4 S/N=12.3
Time
%

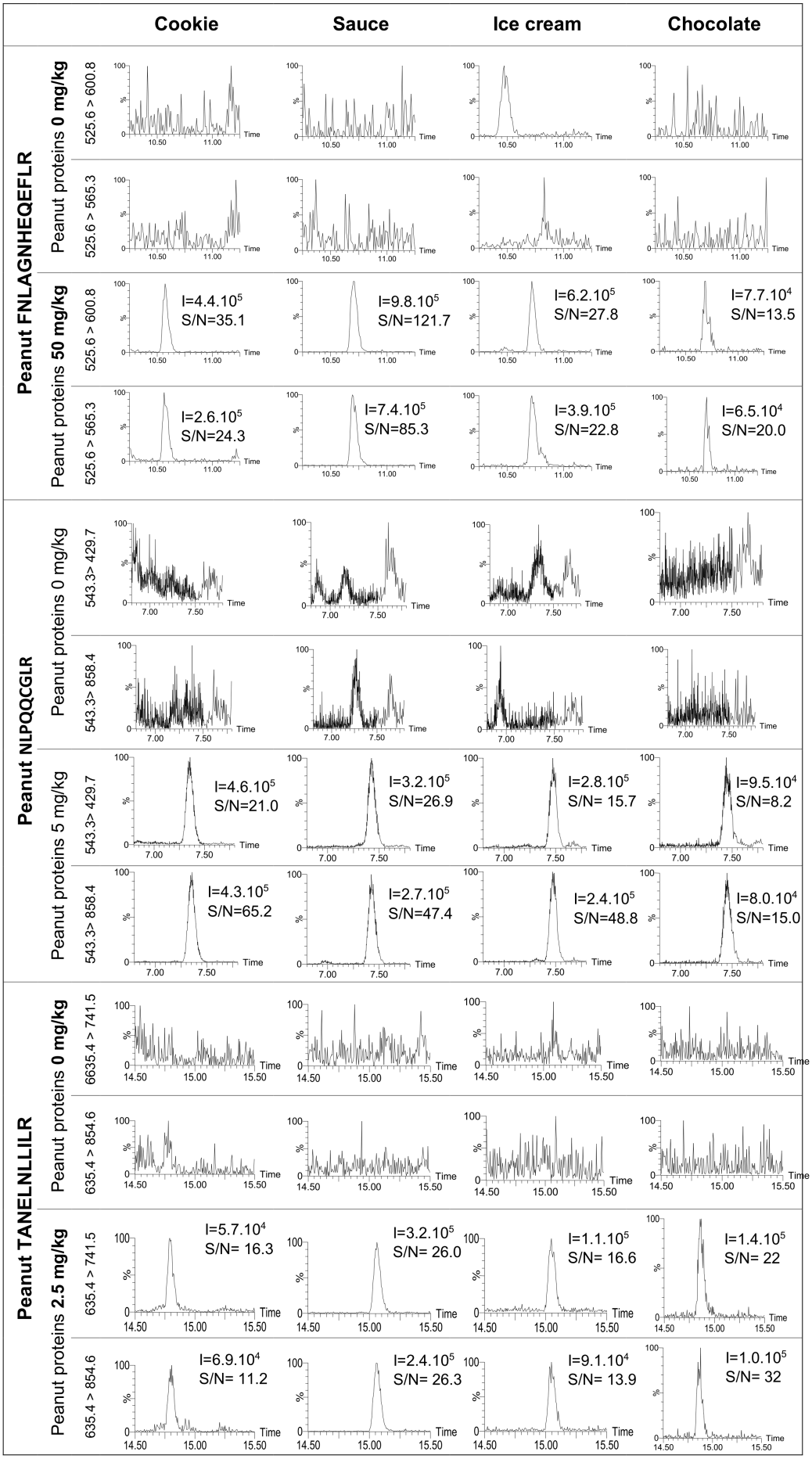

Cookie
Sauce
Ice cream
Chocolate
Walnut ATLTLVSQETR
Walnut prot. 0 mg/kg
609.8> 620.3
6809.8> 719.4
Walnut prot 12.5 mg/kg
609.8> 620.3
I=2.5.10^5 S/N=17.8
I=3.5.10^5 S/N=14.7
I=2.6.10^5 S/N=10.4
I=1.7.10^5 S/N=16.8
6809.8> 719.4
I=2.7.10^5 S/N=19.3
I=3.5.10^5 S/N=14.6
I=2.7.10^5 S/N=21.9
I=1.8.10^5 S/N=18.8
Pecan nuts and walnut LLQPVNNPGQFR
Walnut prot. 0 mg/kg
691.9> 1028.5
691.9> 604.3
Walnut prot 5 mg/kg
691.9> 1028.5
I=6.0.10^5 S/N=26.2
I=5.5.10^5 S/N=43.8
I=8.0.10^5 S/N= 67.4
I=1.2.10^5 S/N=26.5
691.9> 604.3
I=2.6.10^5 S/N=9.4
I=2.4.10^5 S/N=29.6
I=4.1.10^5 S/N=36.0
I=4.3.10^4 S/N=3.2
Pecan nut and walnut VFSNDILVAALNTPR
Walnut prot. 0 mg/kg
815.5> 954.6
815.5> 841.5
Walnut prot 2.5 mg/kg
815.5> 954.6
I=1.4.10^5 S/N=16.5
I=2.5.10^5 S/N=40.4
I=3.9.10^5 S/N=22.2
I=9.7.10^4 S/N=25.3
815.5> 841.5
I=1.7.10^5 S/N=17.8
I=2.4.10^5 S/N=33.4
I=3.4.10^5 S/N=42.6
I=7.8.10^4 S/N=25.5

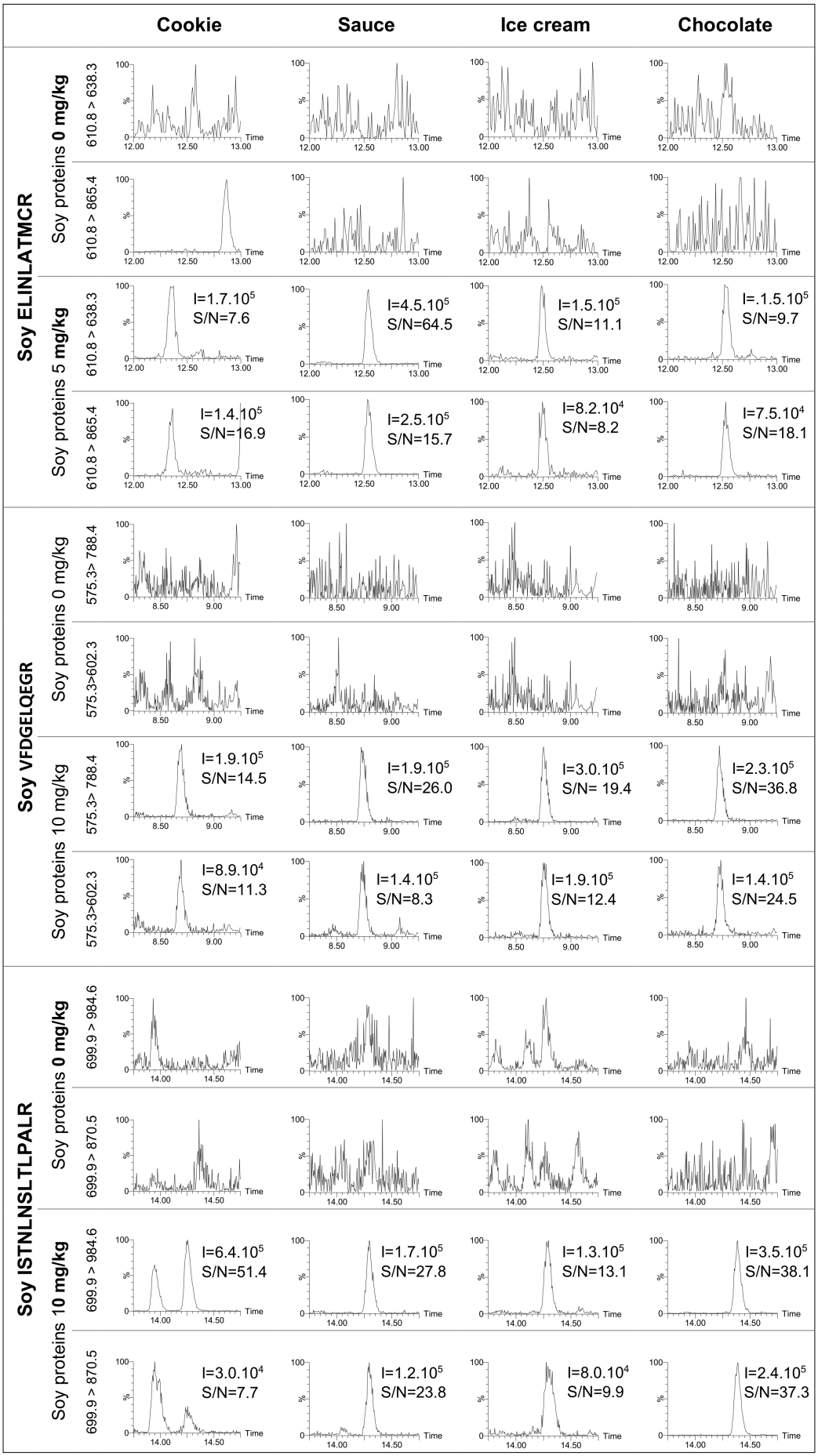

Cookie
Sauce
Ice cream
Chocolate
Pecan nut ATLTFVSQER
Pecan nut prot. 0 mg/kg
576.3 > 618.3
576.3> 765.4
Pecan nut prot 12.5 mg/kg
I=3.0.10^5 S/N=13.3
I=5.0.10^5 S/N=30.3
I=1.7.10^5 S/N=15.5
I=7.0.10^4 S/N=9.1
I=2.7.10^5 S/N=35.7
I=4.8.10^5 S/N=20.7
I=1.2.10^5 S/N=13.9
I=7.6.10^4 S/N=14.4
Pecan nut NFLAGQNNIINQLER
582.0> 659.4
582.0> 772.4
Pecan nut prot 12.5 mg /kg
I=4.7.10^4 S/N=7.8
I=4.1.10^5 S/N=33.9
I=2.0.10^5 S/N=24.2
I=4.7.10^4 S/N=7.8
I=9.1.10^4 S/N=15.4
I=5.3.10^5 S/N=43.4
I=2.9.10^5 S/N=44.2
I=9.1.10^4 S/N=15.4
Pecan nut QVESYFVPMER
692.8> 532.3
692.8> 778.4
Pecan nut prot 25 mg/kg
I=8.7.10^5 S/N=33.3
I=1.0.10^6 S/N=55.5
I=3.6.10^5 S/N=14.1
I=2.0.10^5 S/N=9.9
I=3.8.10^5 S/N=15.3
I=4.5.10^5 S/N=28.3
I=1.5.10^5 S/N=6.5
I=8.9.10^4 S/N=13.6

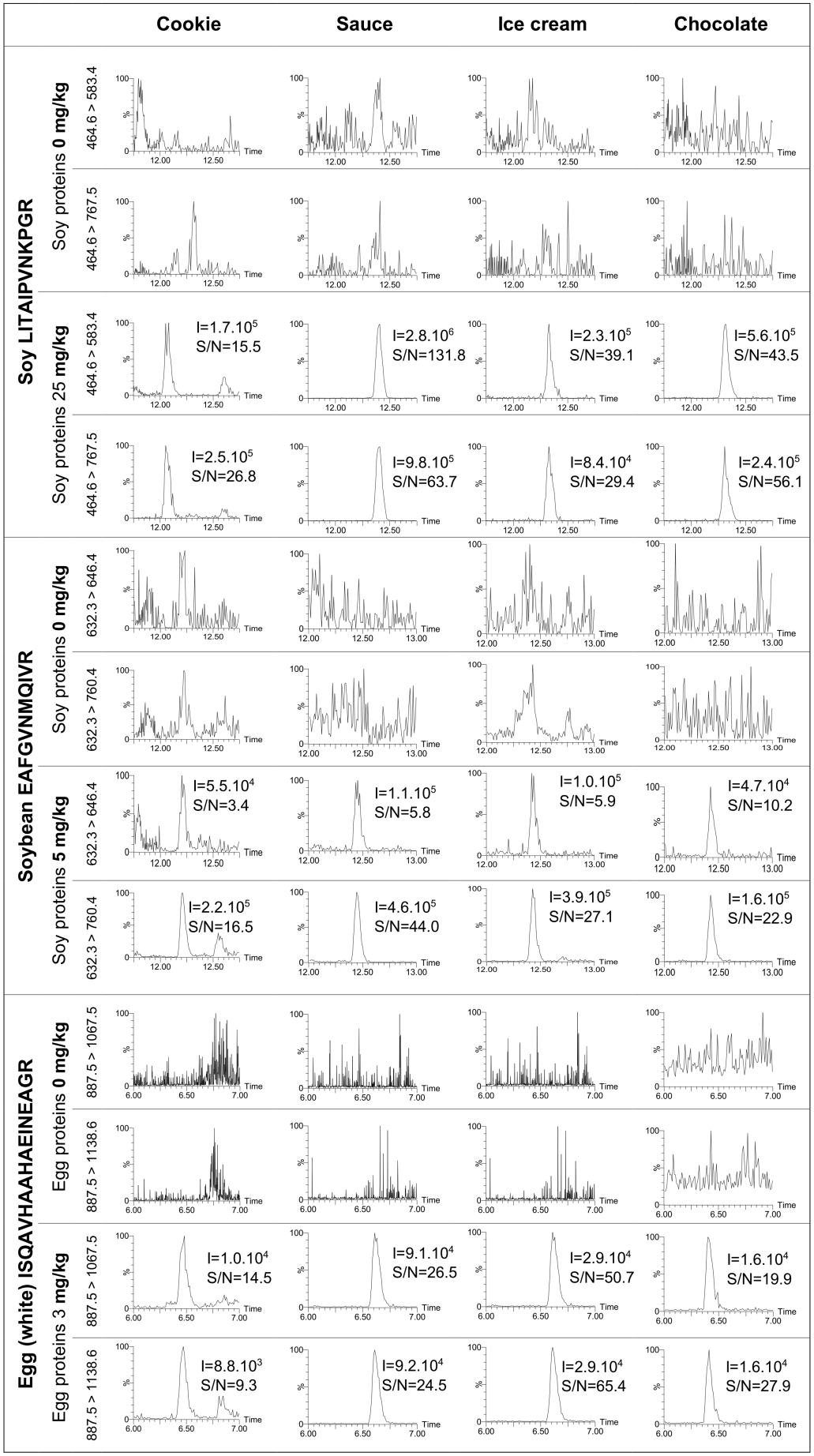

Cookie
Sauce
Ice cream
Chocolate
Pecan nut LVGFGGINGK
Pecan nut prot. 0 mg/kg
452.8> 692.4
452.8> 488.3
Pecan nut prot 5 mg/kg
I=6.7.10^5 S/N=33.8
I=7.8.10^5 S/N=24.8
I=6.8.10^5 S/N=23.9
I=1.3.10^5 S/N=20.2
I=9.7.10^4 S/N=4.8
I=1.1.10^5 S/N=6.5
I=8.0.10^4 S/N=8.7
I=3.5.10^4 S/N=3.8
Milk (casein) YLGYLEQLLR
Milk proteins 0 mg/kg
634.4 > 771.5
634.4 > 934.5
Milk proteins 0.5 mg/kg
I=4.8.10^4 S/N=10.0
I=3.5.10^5 S/N=46.9
I=2.9.10^5 S/N=32.6
I=1.1.10^5 S/N=23.0
I=1.4.10^4 S/N=6.0
I=1.0.10^5 S/N=19.1
I=8.9.10^4 S/N=17.2
I=3.6.10^4 S/N=13.0
Milk (casein) NAVPITPTLNR
Milk proteins 0 mg/kg
598.3 > 285.2
598.3 > 911.5
Milk proteins 2.5 mg/kg
I=1.5.10^5 S/N=13.2
I=1.3.10^6 S/N=57.1
I=2.7.10^5 S/N=15.4
I=6.8.10^5 S/N=31.2
I=1.2.10^5 S/N=9.7
I=9.9.10^5 S/N=125.9
I=1.8.10^5 S/N=25.9
I=5.2.10^5 S/N=61.1
Time

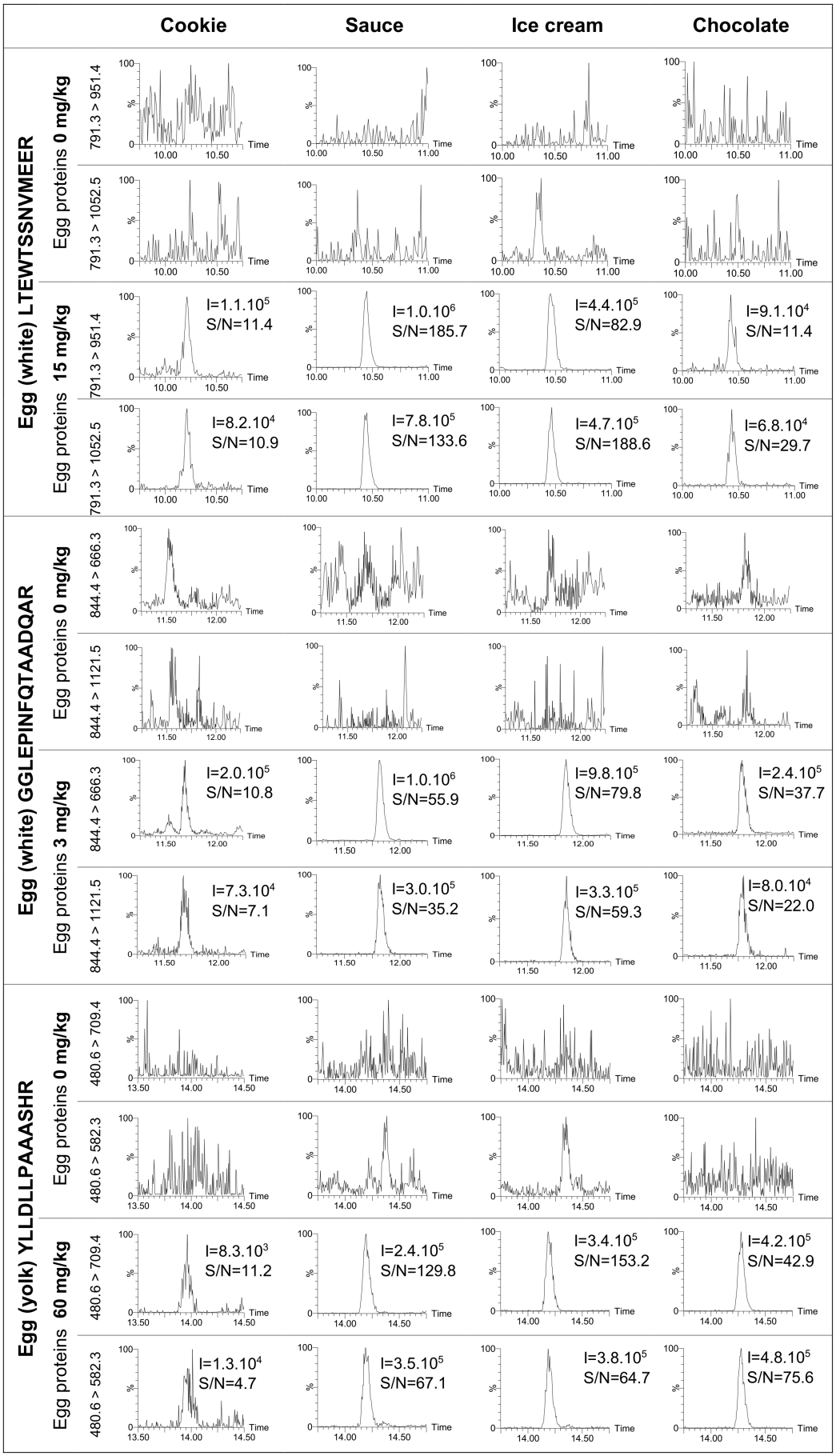

Cookie
Sauce
Ice cream
Chocolate
Milk (casein) HQGLPQEVLNENLLR
Milk proteins 0 mg/kg
587.3 > 436.2
587.3 > 758.4
Milk proteins 5 mg/kg
587.3 > 436.2
I=2.1.10^5 S/N=24.4
I=2.8.10^6 S/N= 20.6
I=1.3.10^5 S/N=10.1
I=4.5.10^5 S/N=14.5
587.3 > 758.4
I=1.2.10^5 S/N=11.5
I=2.2.10^5 S/N=23.7
I=8.1.10^4 S/N=3.8
I=2.7.10^5 S/N=19.1
Milk (casein) FFVAPFPEVFGK
Milk proteins 0 mg/kg
692.9 > 920.5
692.9 > 991.5
Milk proteins 0.5 mg/kg
692.9 > 920.5
I=1.4.10^5 S/N=21.5
I=1.5.10^6 S/N=268.
I=9.2.10^5 S/N=143.3
I=1.5.10^5 S/N=44.0
692.9 > 991.5
I=6.3.10^4 S/N=12.9
I=5.0.10^5 S/N=129.1
I=3.1.10^5 S/N=65.2
I=5.9.10^4 S/N=13.3
Milk (whey) VYVEELKPTPEGDLEILLQK
Milk proteins 0 mg/kg
771.8 > 627.9
771.8 >790.9
Milk proteins 5 mg/kg
771.8 > 627.9
S/N<3
I=1.6.10^5 S/N=31.3
I=3.5.10^4 S/N=8.4
I=9.4.10^4 S/N=17.4
771.8 >790.9
S/N<3
I=1.1.10^5 S/N=28.1
I=5.1.10^4 S/N=10.2
I=6.3.10^4 S/N=20.4

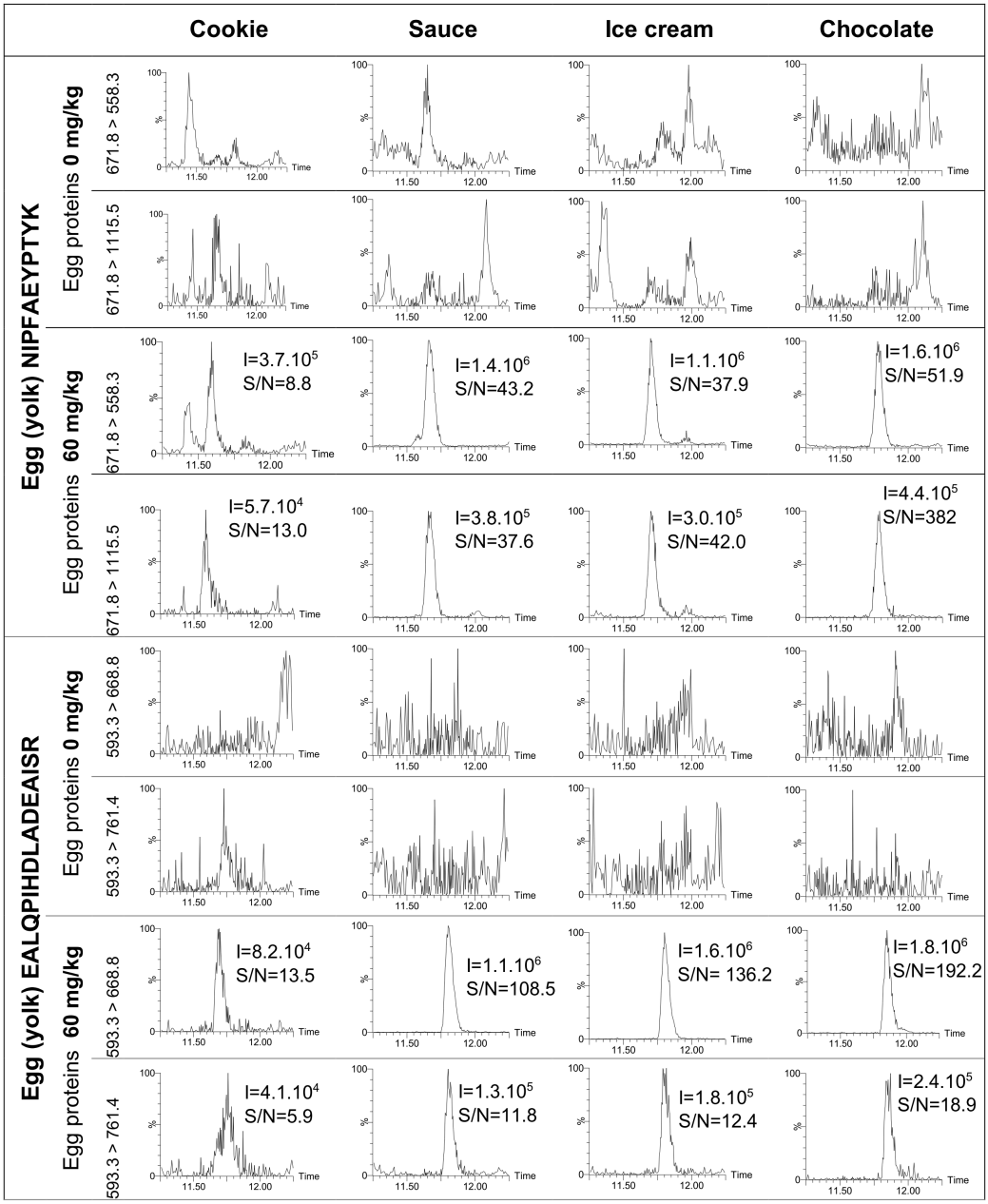

Cookie
Sauce
Ice cream
Chocolate
Milk (whey) LSFNPTQLEEQCHI
Milk proteins 0 mg/kg
858.4 > 627.8
858.4 > 1254.6
Milk proteins 5 mg/kg
858.4 > 627.8
I=3.8.10^4
S/N=4.6
I=3.1.10^5
S/N=23.4
I=2.5.10^5
S/N=36.0
I=3.5.10^5
S/N=34.7
858.4 > 1254.6
I=3.2.10^4
S/N=13.1
I=3.8.10^5
S/N=93.4
I=2.6.10^5
S/N=42.0
I=4.2.10^5
S/N=102.5
Milk (whey) VLVLVTDYK
Milk proteins 0 mg/kg
533.3 > 754.4
533.3 > 853.4
Milk proteins 10 mg/kg
533.3 > 754.4
I=3.5.10^4
S/N=4.3
I=6.9.10^5
S/N=42.7
I=2.6.10^5
S/N=20.5
I=3.2.10^5
S/N=52.9
533.3 > 853.4
I=6.8.10^4
S/N=10
I=1.3.10^6
S/N=214.1
I=4.1.10^5
S/N=36.8
I=5.8.10^5
S/N=147.4
Peanut RPYSNAPQEIFIQQGR
Peanut proteins 0 mg/kg
684.4 >748.4
684.4 > 836.4
Peanut proteins 5 mg/kg
684.4 >748.4
I=4.9.10^5
S/N=38.8
I=2.3.10^5
S/N=25.7
I=4.9.10^5
S/N=27.1
S/N<3
684.4 > 836.4
I=3.6.10^5
S/N=21.4
I=1.6.10^5
S/N=11.0
I=4.0.10^5
S/N=19.6
S/N<3


Cookie
Sauce
Ice cream
Chocolate
Peanut FNLAGNHEQEFLR
Peanut proteins 0 mg/kg
525.6 > 600.8
525.6 > 565.3
Peanut proteins 50 mg/kg
525.6 > 600.8
I=4.4.10^5 S/N=35.1
I=9.8.10^5 S/N=121.7
I=6.2.10^5 S/N=27.8
I=7.7.10^4 S/N=13.5
525.6 > 565.3
I=2.6.10^5 S/N=24.3
I=7.4.10^5 S/N=85.3
I=3.9.10^5 S/N=22.8
I=6.5.10^4 S/N=20.0
Peanut NLPQQCGLR
Peanut proteins 0 mg/kg
543.3> 429.7
543.3> 858.4
Peanut proteins 5 mg/kg
543.3> 429.7
I=4.6.10^5 S/N=21.0
I=3.2.10^5 S/N=26.9
I=2.8.10^5 S/N= 15.7
I=9.5.10^4 S/N=8.2
543.3> 858.4
I=4.3.10^5 S/N=65.2
I=2.7.10^5 S/N=47.4
I=2.4.10^5 S/N=48.8
I=8.0.10^4 S/N=15.0
Peanut TANELNLLILR
Peanut proteins 0 mg/kg
6635.4 > 741.5
635.4 > 854.6
Peanut proteins 2.5 mg/kg
635.4 > 741.5
I=5.7.10^4 S/N= 16.3
I=3.2.10^5 S/N= 26.0
I=1.1.10^5 S/N= 16.6
I=1.4.10^5 S/N= 22
635.4 > 854.6
I=6.9.10^4 S/N= 11.2
I=2.4.10^5 S/N= 26.3
I=9.1.10^4 S/N= 13.9
I=1.0.10^5 S/N= 32

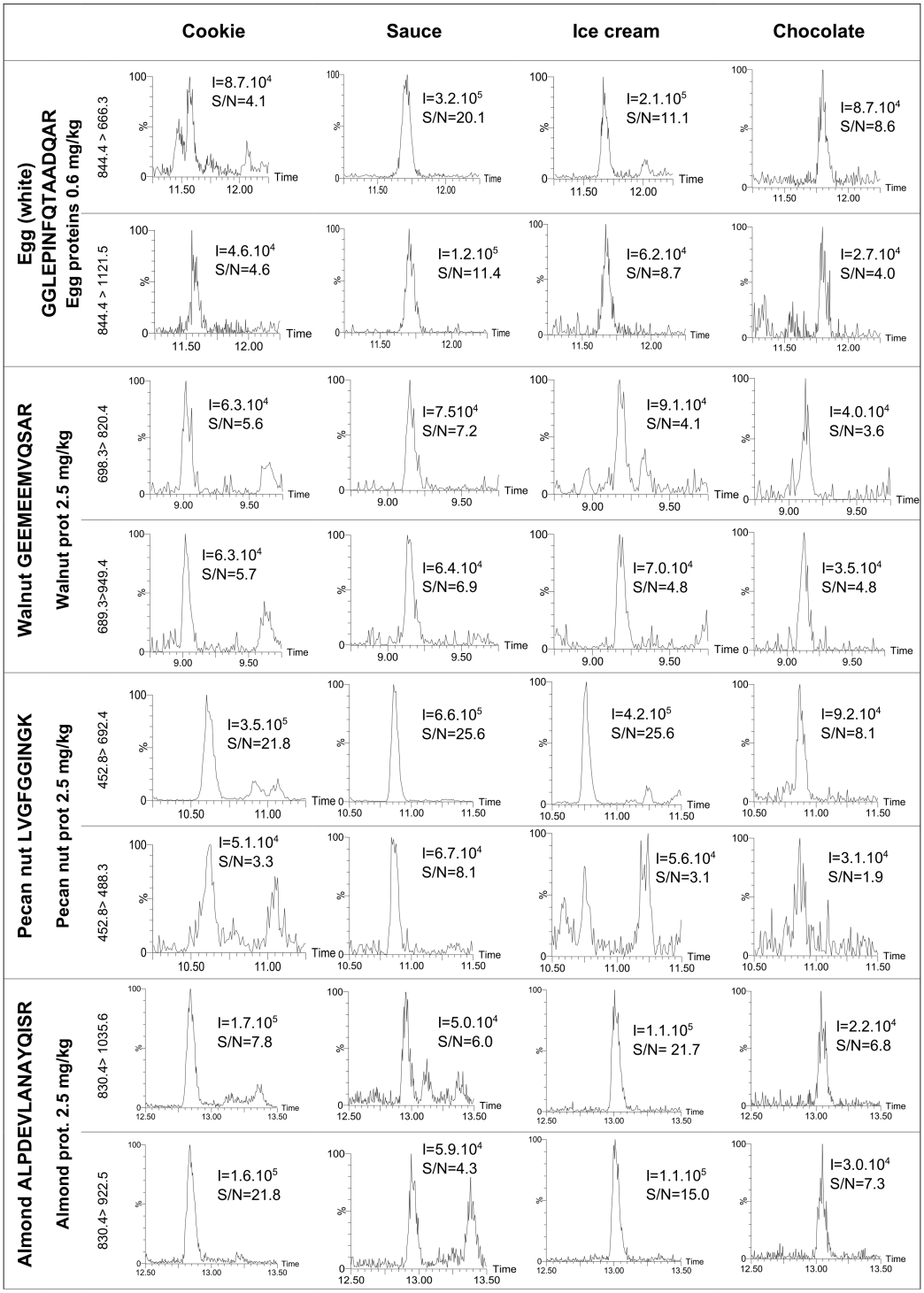

Cookie
Sauce
Ice cream
Chocolate
Soy ELINLATMCR
Soy proteins 0 mg/kg
610.8 > 638.3
610.8 > 865.4
Soy proteins 5 mg/kg
610.8 > 638.3
I=1.7.10^5 S/N=7.6
I=4.5.10^5 S/N=64.5
I=1.5.10^5 S/N=11.1
I=.1.5.10^5 S/N=9.7
610.8 > 865.4
I=1.4.10^5 S/N=16.9
I=2.5.10^5 S/N=15.7
I=8.2.10^4 S/N=8.2
I=7.5.10^4 S/N=18.1
Soy VFDGELQEGR
Soy proteins 0 mg/kg
575.3> 788.4
575.3>602.3
Soy proteins 10 mg/kg
575.3> 788.4
I=1.9.10^5 S/N=14.5
I=1.9.10^5 S/N=26.0
I=3.0.10^5 S/N= 19.4
I=2.3.10^5 S/N=36.8
575.3>602.3
I=8.9.10^4 S/N=11.3
I=1.4.10^5 S/N=8.3
I=1.9.10^5 S/N=12.4
I=1.4.10^5 S/N=24.5
Soy ISTNLNSLTLPALR
Soy proteins 0 mg/kg
699.9 > 984.6
699.9 > 870.5
Soy proteins 10 mg/kg
699.9 > 984.6
I=6.4.10^5 S/N=51.4
I=1.7.10^5 S/N=27.8
I=1.3.10^5 S/N=13.1
I=3.5.10^5 S/N=38.1
699.9 > 870.5
I=3.0.10^4 S/N=7.7
I=1.2.10^5 S/N=23.8
I=8.0.10^4 S/N=9.9
I=2.4.10^5 S/N=37.3


Cookie
Sauce
Ice cream
Chocolate
Soy LITAIPVNKPGR
Soy proteins 0 mg/kg
464.6 > 583.4
464.6 > 767.5
Soy proteins 25 mg/kg
I=1.7.10^5 S/N=15.5
I=2.8.10^6 S/N=131.8
I=2.3.10^5 S/N=39.1
I=5.6.10^5 S/N=43.5
I=2.5.10^5 S/N=26.8
I=9.8.10^5 S/N=63.7
I=8.4.10^4 S/N=29.4
I=2.4.10^5 S/N=56.1
Soybean EAFGVNMQIVR
Soy proteins 0 mg/kg
632.3 > 646.4
632.3 > 760.4
Soy proteins 5 mg/kg
I=5.5.10^4 S/N=3.4
I=1.1.10^5 S/N=5.8
I=1.0.10^5 S/N=5.9
I=4.7.10^4 S/N=10.2
I=2.2.10^5 S/N=16.5
I=4.6.10^5 S/N=44.0
I=3.9.10^5 S/N=27.1
I=1.6.10^5 S/N=22.9
Egg (white) ISQAVHAAHAEINEAGR
Egg proteins 0 mg/kg
887.5 > 1067.5
887.5 > 1138.6
Egg proteins 3 mg/kg
I=1.0.10^4 S/N=14.5
I=9.1.10^4 S/N=26.5
I=2.9.10^4 S/N=50.7
I=1.6.10^4 S/N=19.9
I=8.8.10^3 S/N=9.3
I=9.2.10^4 S/N=24.5
I=2.9.10^4 S/N=65.4
I=1.6.10^4 S/N=27.9

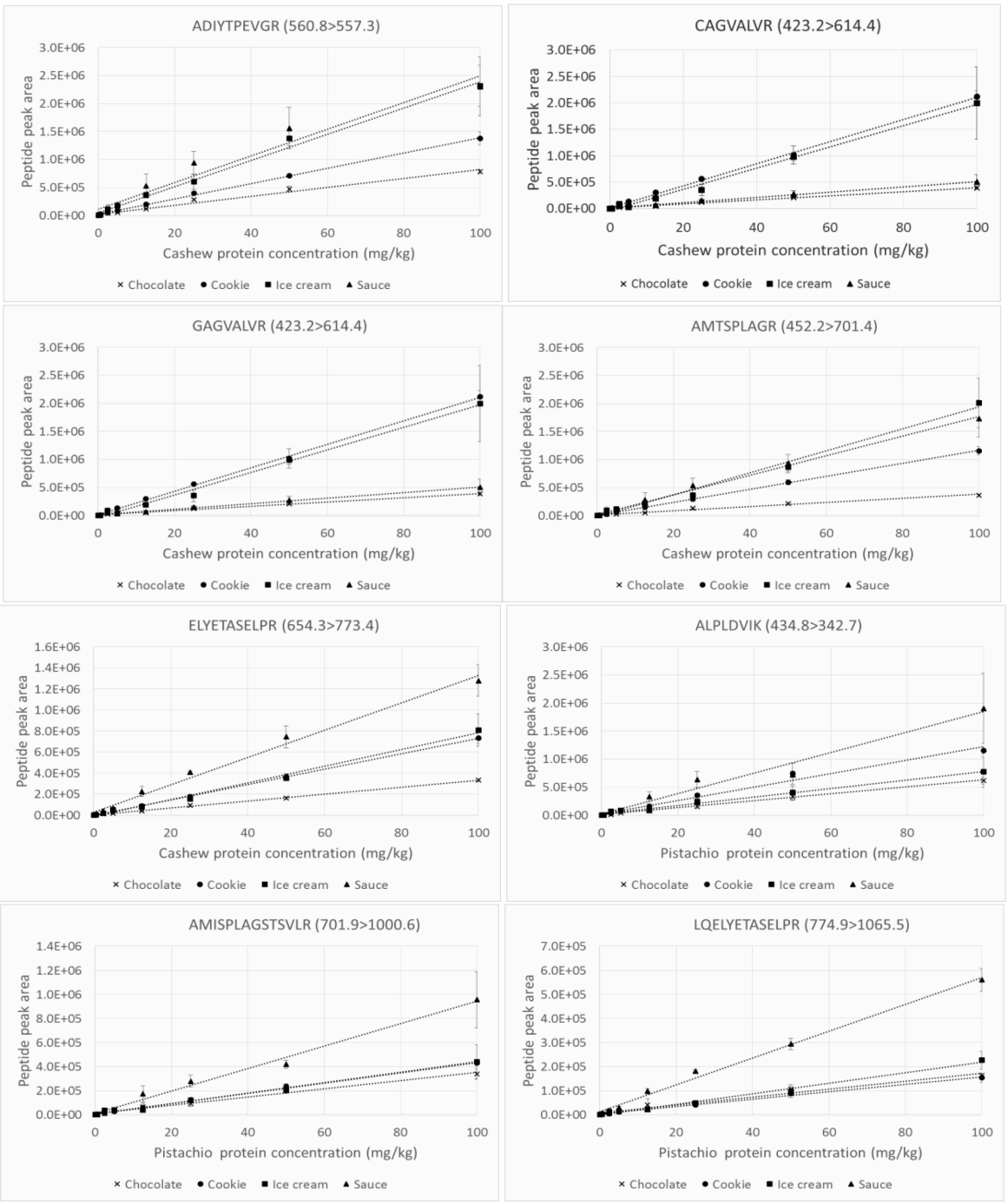

Cookie
Sauce
Ice cream
Chocolate
Egg (white) LTEWTSSNVMEER
Egg proteins 0 mg/kg
791.3 > 951.4
791.3 > 1052.5
Egg proteins 15 mg/kg
I=1.1.10^5 S/N=11.4
I=1.0.10^6 S/N=185.7
I=4.4.10^5 S/N=82.9
I=9.1.10^4 S/N=11.4
I=8.2.10^4 S/N=10.9
I=7.8.10^5 S/N=133.6
I=4.7.10^5 S/N=188.6
I=6.8.10^4 S/N=29.7
Egg (white) GGLEPINFQTAADQAR
Egg proteins 0 mg/kg
844.4 > 666.3
844.4 > 1121.5
Egg proteins 3 mg/kg
I=2.0.10^5 S/N=10.8
I=1.0.10^6 S/N=55.9
I=9.8.10^5 S/N=79.8
I=2.4.10^5 S/N=37.7
I=7.3.10^4 S/N=7.1
I=3.0.10^5 S/N=35.2
I=3.3.10^5 S/N=59.3
I=8.0.10^4 S/N=22.0
Egg (yolk) YLLDLLPAASHR
Egg proteins 0 mg/kg
480.6 > 709.4
480.6 > 582.3
Egg proteins 60 mg/kg
I=8.3.10^3 S/N=11.2
I=2.4.10^5 S/N=129.8
I=3.4.10^5 S/N=153.2
I=4.2.10^5 S/N=42.9
I=1.3.10^4 S/N=4.7
I=3.5.10^5 S/N=67.1
I=3.8.10^5 S/N=64.7
I=4.8.10^5 S/N=75.6

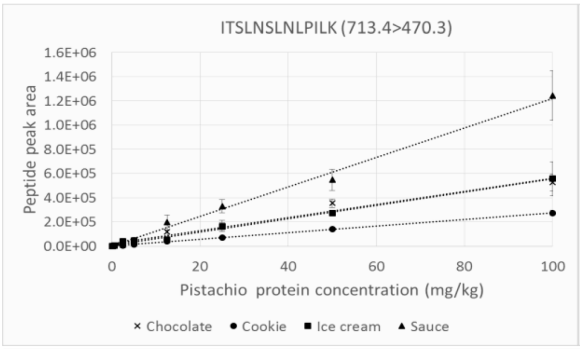

Cookie
Sauce
Ice cream
Chocolate
Egg (yolk) NIPFAEYPTYK
Egg proteins 0 mg/kg
671.8 > 558.3
671.8 > 1115.5
Egg proteins 60 mg/kg
671.8 > 558.3
I=3.7.10^5
S/N=8.8
I=1.4.10^6
S/N=43.2
I=1.1.10^6
S/N=37.9
I=1.6.10^6
S/N=51.9
671.8 > 1115.5
I=5.7.10^4
S/N=13.0
I=3.8.10^5
S/N=37.6
I=3.0.10^5
S/N=42.0
I=4.4.10^5
S/N=382
Egg (yolk) EALQPIHDLADEAISR
Egg proteins 0 mg/kg
593.3 > 668.8
593.3 > 761.4
Egg proteins 60 mg/kg
593.3 > 668.8
I=8.2.10^4
S/N=13.5
I=1.1.10^6
S/N=108.5
I=1.6.10^6
S/N= 136.2
I=1.8.10^6
S/N=192.2
593.3 > 761.4
I=4.1.10^4
S/N=5.9
I=1.3.10^5
S/N=11.8
I=1.8.10^5
S/N=12.4
I=2.4.10^5
S/N=18.9
Time
11.50
12.00

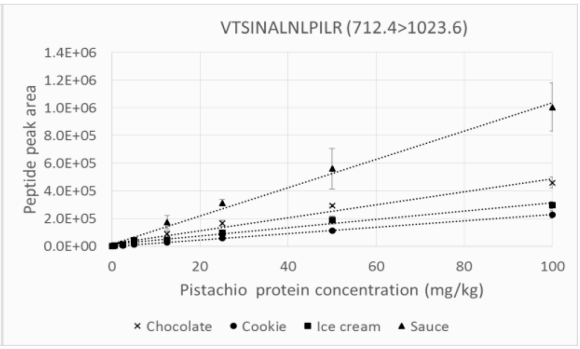
Complementary figure 2: Chromatograms of the two highest multiple reaction monitoring MRM transitions of egg, walnut, pecan nut, and almond allergens. Data for processed matrices at the limit of detection (LOD) are presented without smoothing. The signal-to-noise ratio (S/N) determined in peak-to-peak mode and the peak absolute intensity (I) are indicated for each transition at the LOQ level.

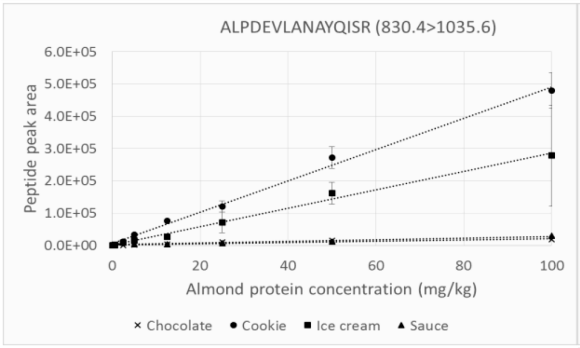


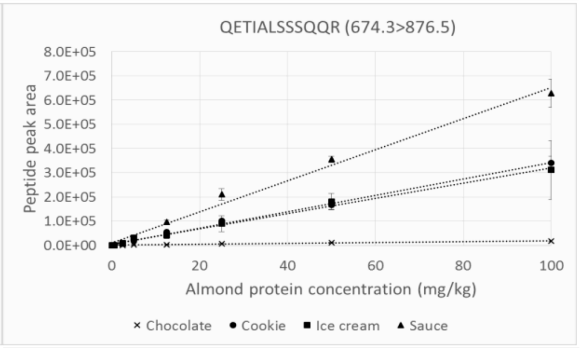
Complementary figure 3-a : Linear regression of peptide peak area of the most abundant MRM transition as a function of the concentration of allergen proteins for tree nuts (cashew, pistachio, almond, hazelnut, pecan, and walnut). Linearity was checked by analyzing three independent preparations of sauce, ice cream, chocolate, and cookies at 8 levels of concentration (complementary data).

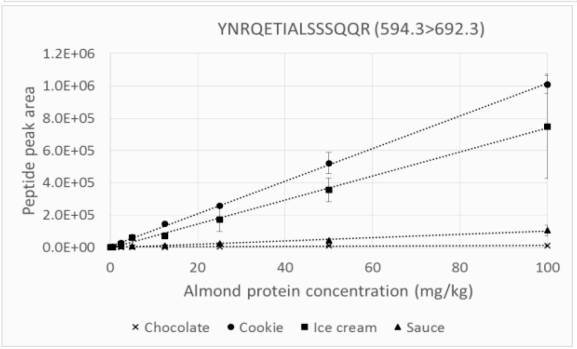


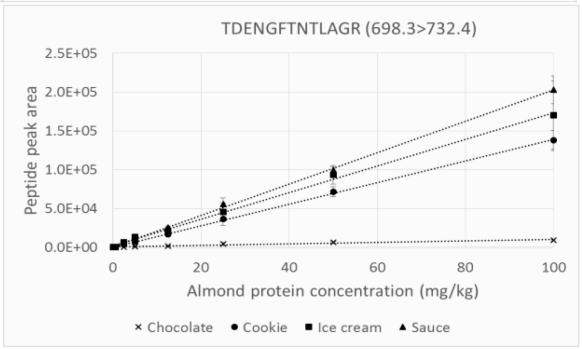

ITSLNSLNLPILK (713.4>470.3)
Peptide peak area
Pistachio protein concentration (mg/kg)
Chocolate
Cookie
Ice cream
Sauce

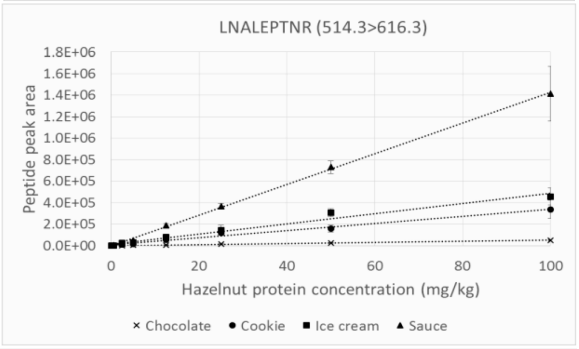

VTSINALNLPILR (712.4>1023.6)
Peptide peak area
Pistachio protein concentration (mg/kg)
Chocolate
Cookie
Ice cream
Sauce

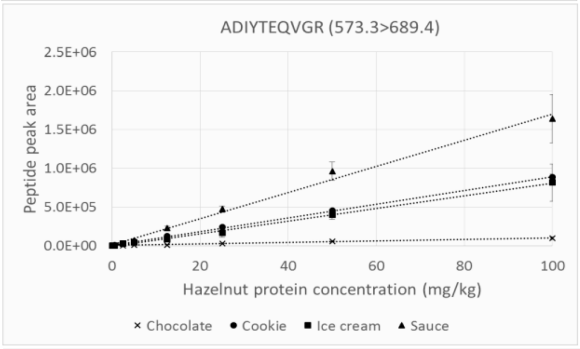

ALPDEVLANAYQISR (830.4>1035.6)
Peptide peak area
Almond protein concentration (mg/kg)
Chocolate
Cookie
Ice cream
Sauce

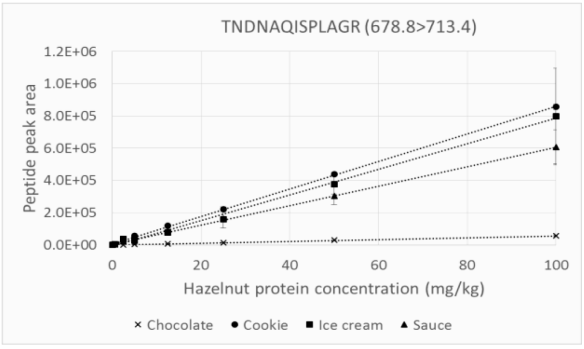

QETIALSSSQQR (674.3>876.5)
Peptide peak area
Almond protein concentration (mg/kg)
Chocolate
Cookie
Ice cream
Sauce

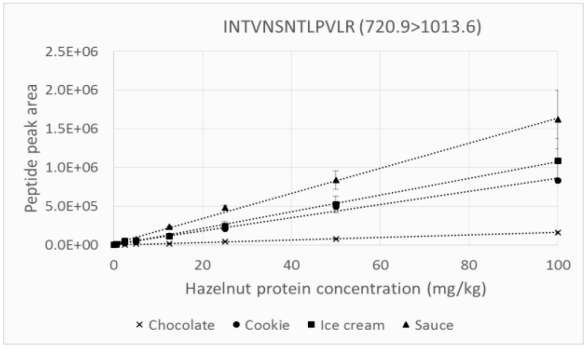

YNRQETIALSSSQQR (594.3>692.3)
Peptide peak area
Almond protein concentration (mg/kg)
Chocolate
Cookie
Ice cream
Sauce

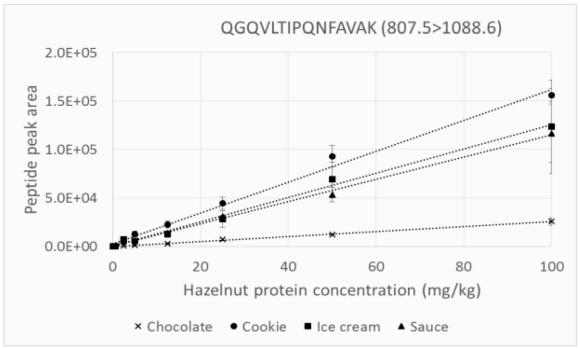

TDENGFTNTLAGR (698.3>732.4)
Peptide peak area
Almond protein concentration (mg/kg)
Chocolate
Cookie
Ice cream
Sauce

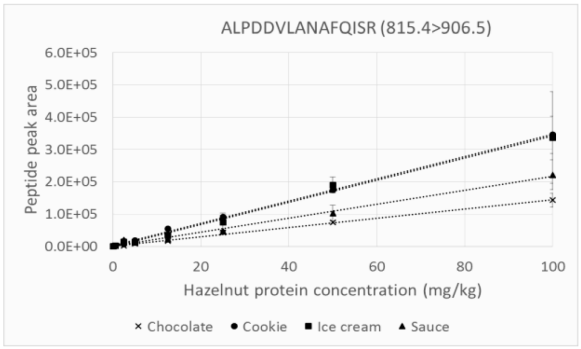

LNALEPTNR (514.3>616.3)
Peptide peak area
Hazelnut protein concentration (mg/kg)
Chocolate
Cookie
Ice cream
Sauce

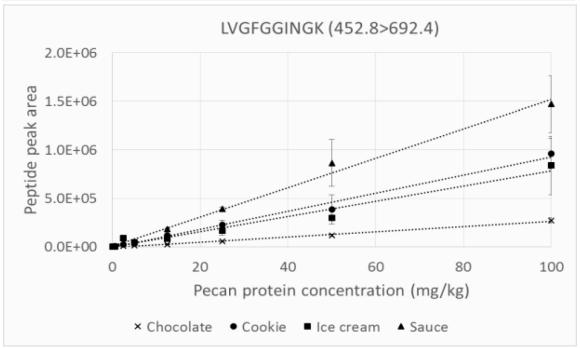

ADIYTEQVGR (573.3>689.4)
Peptide peak area
Hazelnut protein concentration (mg/kg)
Chocolate
Cookie
Ice cream
Sauce

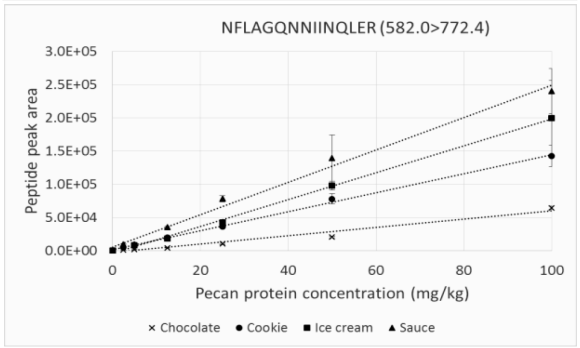

TNDNAQISPLAGR (678.8>713.4)
Peptide peak area
Hazelnut protein concentration (mg/kg)
Chocolate
Cookie
Ice cream
Sauce

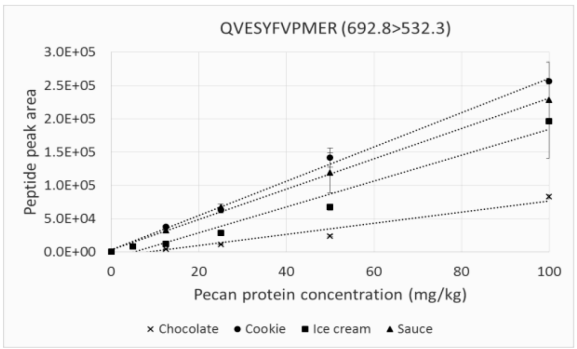

INTVNSNTLPVLR (720.9>1013.6)
Peptide peak area
Hazelnut protein concentration (mg/kg)
Chocolate
Cookie
Ice cream
Sauce

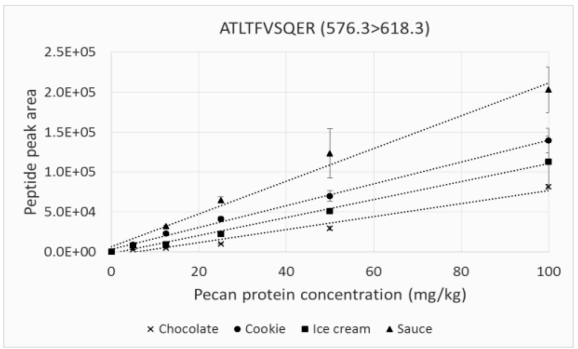

QGQVLTIPQNFAVAK (807.5>1088.6)
Peptide peak area
2.0E+05
1.5E+05
1.0E+05
5.0E+04
0.0E+00
0
20
40
60
80
100
Hazelnut protein concentration (mg/kg)
× Chocolate ● Cookie ■ Ice cream ▲ Sauce

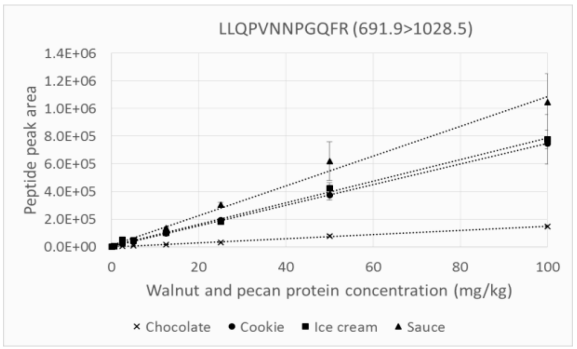

ALPDDVLANAFQISR (815.4>906.5)
Peptide peak area
6.0E+05
5.0E+05
4.0E+05
3.0E+05
2.0E+05
1.0E+05
0.0E+00
0
20
40
60
80
100
Hazelnut protein concentration (mg/kg)
× Chocolate ● Cookie ■ Ice cream ▲ Sauce

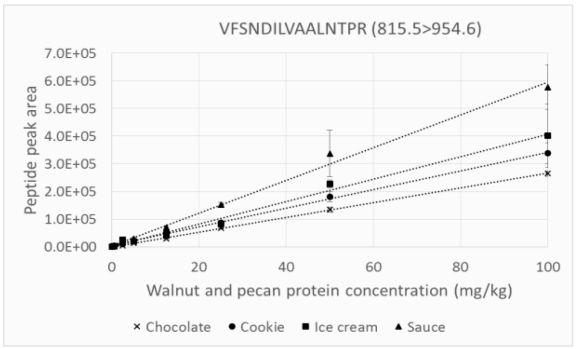

LVGFGGINGK (452.8>692.4)
Peptide peak area
2.0E+06
1.5E+06
1.0E+06
5.0E+05
0.0E+00
0
20
40
60
80
100
Pecan protein concentration (mg/kg)
× Chocolate ● Cookie ■ Ice cream ▲ Sauce

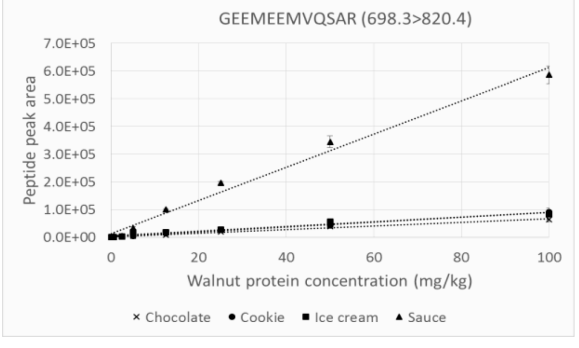

NFLAGQNNIINQLER (582.0>772.4)
Peptide peak area
3.0E+05
2.5E+05
2.0E+05
1.5E+05
1.0E+05
5.0E+04
0.0E+00
0
20
40
60
80
100
Pecan protein concentration (mg/kg)
× Chocolate ● Cookie ■ Ice cream ▲ Sauce

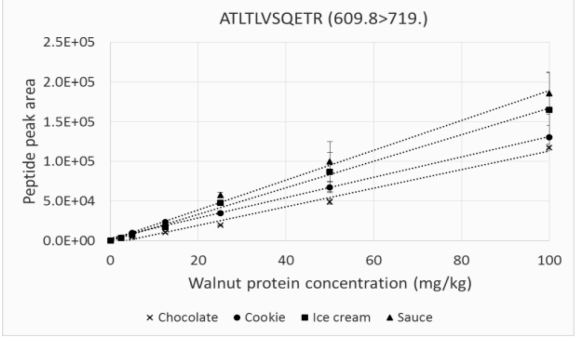

QVESYFVPMER (692.8>532.3)
Peptide peak area
3.0E+05
2.5E+05
2.0E+05
1.5E+05
1.0E+05
5.0E+04
0.0E+00
0
20
40
60
80
100
Pecan protein concentration (mg/kg)
× Chocolate ● Cookie ■ Ice cream ▲ Sauce


ATLTFVSQER (576.3>618.3)
Peptide peak area
2.5E+05
2.0E+05
1.5E+05
1.0E+05
5.0E+04
0.0E+00
0
20
40
60
80
100
Pecan protein concentration (mg/kg)
× Chocolate ● Cookie ■ Ice cream ▲ Sauce

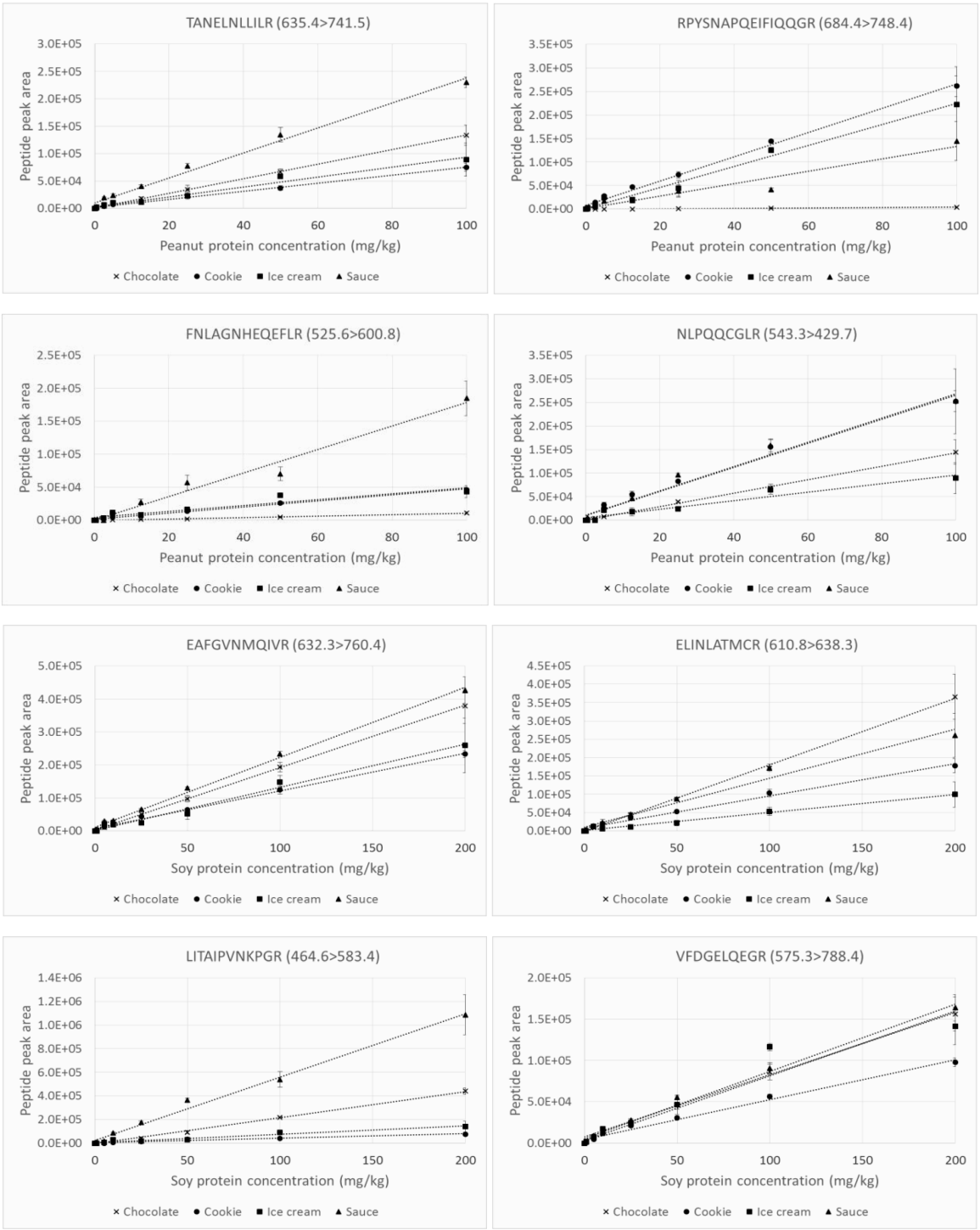

LLQPVNNPGQFR (691.9>1028.5)
Peptide peak area
1.4E+06
1.2E+06
1.0E+06
8.0E+05
6.0E+05
4.0E+05
2.0E+05
0.0E+00
0
20
40
60
80
100
Walnut and pecan protein concentration (mg/kg)
× Chocolate ● Cookie ■ Ice cream ▲ Sauce

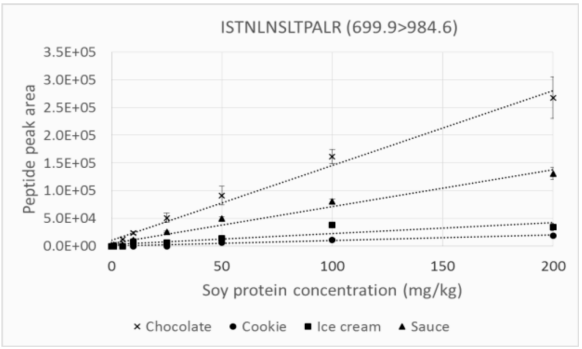

VFSNDILVAALNTPR (815.5>954.6)
Peptide peak area
7.0E+05
6.0E+05
5.0E+05
4.0E+05
3.0E+05
2.0E+05
1.0E+05
0.0E+00
0
20
40
60
80
100
Walnut and pecan protein concentration (mg/kg)
× Chocolate ● Cookie ■ Ice cream ▲ Sauce

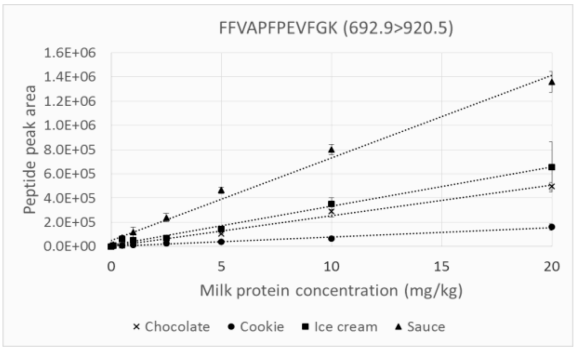

GEEMEEMVQSAR (698.3>820.4)
Peptide peak area
7.0E+05
6.0E+05
5.0E+05
4.0E+05
3.0E+05
2.0E+05
1.0E+05
0.0E+00
0
20
40
60
80
100
Walnut protein concentration (mg/kg)
× Chocolate ● Cookie ■ Ice cream ▲ Sauce

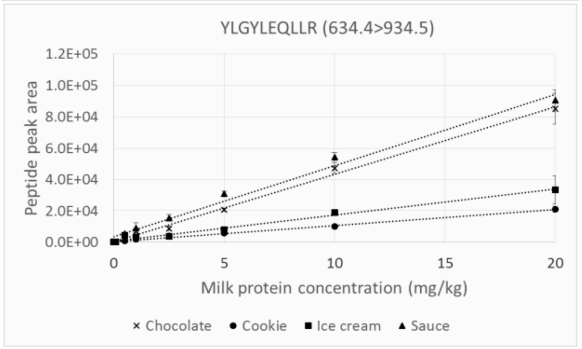

ATLTLVSQETR (609.8>719.)
Peptide peak area
2.5E+05
2.0E+05
1.5E+05
1.0E+05
5.0E+04
0.0E+00
0
20
40
60
80
100
Walnut protein concentration (mg/kg)
× Chocolate ● Cookie ■ Ice cream ▲ Sauce

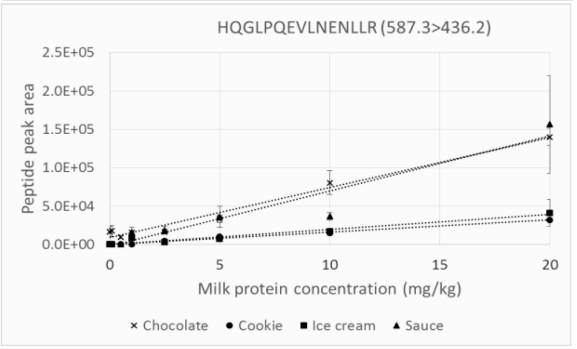
Complementary figure 3-b: Linear regression of peptide peak area of the most abundant MRM transition as a function of the concentration of allergen proteins for peanut, soy, milk and egg. Linearity was checked by analyzing three independent preparations of sauce, ice cream, chocolate, and cookies at 8 levels of concentration (complementary data).

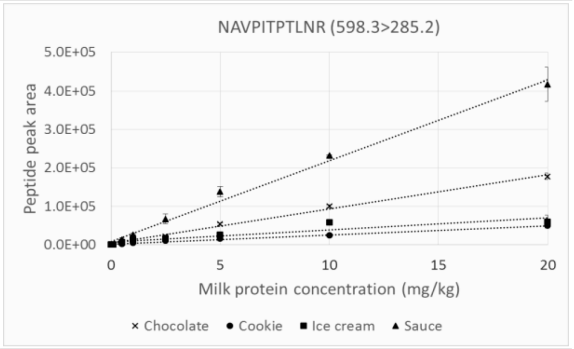


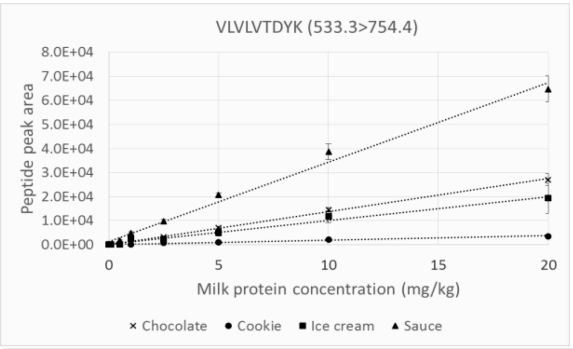

ISTNLNSLTPALR (699.9>984.6)
Peptide peak area
3.5E+05
3.0E+05
2.5E+05
2.0E+05
1.5E+05
1.0E+05
5.0E+04
0.0E+00
0
50
100
150
200
Soy protein concentration (mg/kg)
× Chocolate ● Cookie ■ Ice cream ▲ Sauce

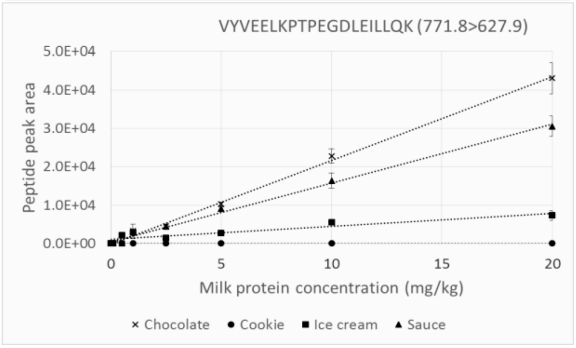

FFVAPFPEVFGK (692.9>920.5)
Peptide peak area
1.6E+06
1.4E+06
1.2E+06
1.0E+06
8.0E+05
6.0E+05
4.0E+05
2.0E+05
0.0E+00
0
5
10
15
20
Milk protein concentration (mg/kg)
× Chocolate ● Cookie ■ Ice cream ▲ Sauce

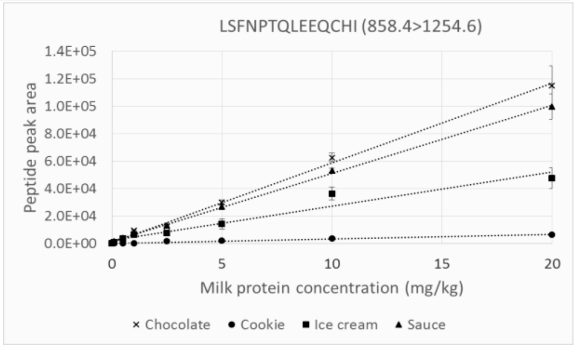

YLGYLEQLLR (634.4>934.5)
Peptide peak area
1.2E+05
1.0E+05
8.0E+04
6.0E+04
4.0E+04
2.0E+04
0.0E+00
0
5
10
15
20
Milk protein concentration (mg/kg)
× Chocolate ● Cookie ■ Ice cream ▲ Sauce

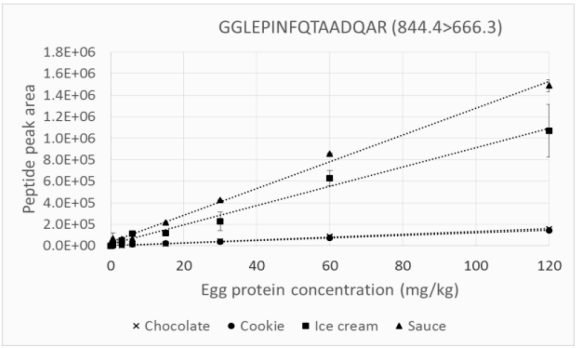

HQGLPQEVLNENLLR (587.3>436.2)
Peptide peak area
2.5E+05
2.0E+05
1.5E+05
1.0E+05
5.0E+04
0.0E+00
0
5
10
15
20
Milk protein concentration (mg/kg)
× Chocolate ● Cookie ■ Ice cream ▲ Sauce

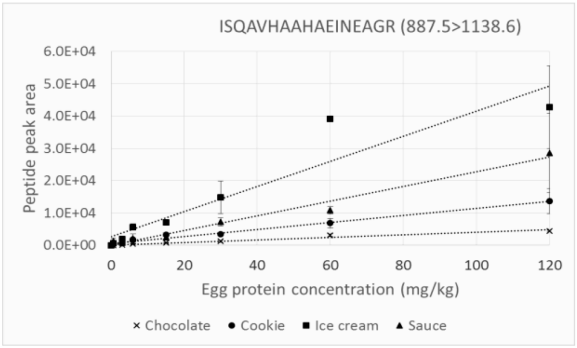

NAVPITPTLNR (598.3>285.2)
Peptide peak area
5.0E+05
4.0E+05
3.0E+05
2.0E+05
1.0E+05
0.0E+00
0
5
10
15
20
Milk protein concentration (mg/kg)
× Chocolate ● Cookie ■ Ice cream ▲ Sauce

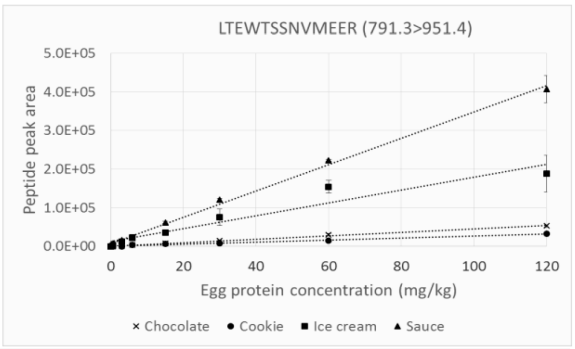

VLVLVTDYK (533.3>754.4)
Peptide peak area
8.0E+04
7.0E+04
6.0E+04
5.0E+04
4.0E+04
3.0E+04
2.0E+04
1.0E+04
0.0E+00
0
5
10
15
20
Milk protein concentration (mg/kg)
× Chocolate ● Cookie ■ Ice cream ▲ Sauce

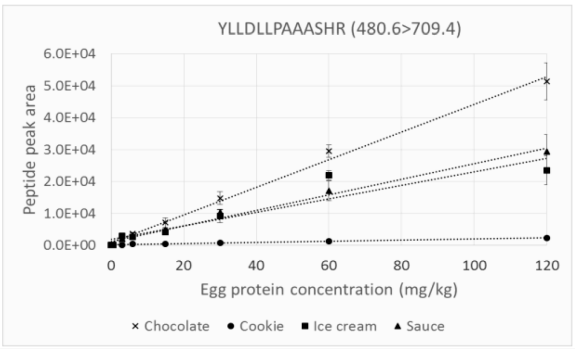

VYVEELKPTPEGDLEILLQK (771.8>627.9)
Peptide peak area
5.0E+04
4.0E+04
3.0E+04
2.0E+04
1.0E+04
0.0E+00
0
5
10
15
20
Milk protein concentration (mg/kg)
× Chocolate ● Cookie ■ Ice cream ▲ Sauce

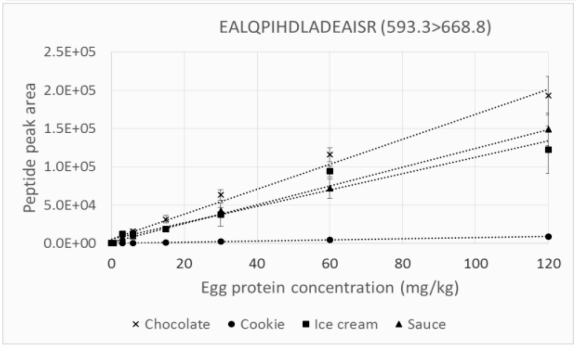

LSFNPTQLEEQCHI (858.4>1254.6)
Peptide peak area
1.4E+05
1.2E+05
1.0E+05
8.0E+04
6.0E+04
4.0E+04
2.0E+04
0.0E+00
0
5
10
15
20
Milk protein concentration (mg/kg)
× Chocolate ● Cookie ■ Ice cream ▲ Sauce

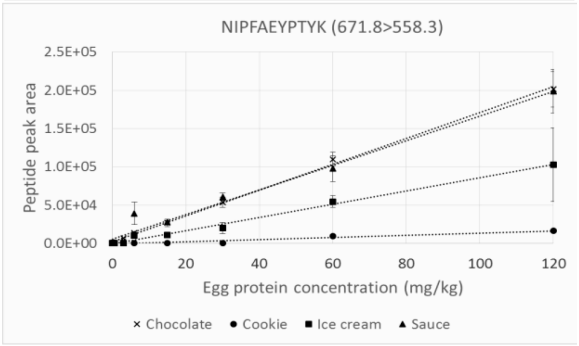

GGLEPINFQTAADQAR (844.4>666.3)
Peptide peak area
1.8E+06
1.6E+06
1.4E+06
1.2E+06
1.0E+06
8.0E+05
6.0E+05
4.0E+05
2.0E+05
0.0E+00
0
20
40
60
80
100
120
Egg protein concentration (mg/kg)
× Chocolate ● Cookie ■ Ice cream ▲ Sauce


ISQAVHAAHAEINEAGR (887.5>1138.6)
Peptide peak area
6.0E+04
5.0E+04
4.0E+04
3.0E+04
2.0E+04
1.0E+04
0.0E+00
0
20
40
60
80
100
120
Egg protein concentration (mg/kg)
× Chocolate ● Cookie ■ Ice cream ▲ Sauce


LTEWTSSNVMEER (791.3>951.4)
Peptide peak area
5.0E+05
4.0E+05
3.0E+05
2.0E+05
1.0E+05
0.0E+00
0
20
40
60
80
100
120
Egg protein concentration (mg/kg)
× Chocolate ● Cookie ■ Ice cream ▲ Sauce


YLLDLLPAAASHR (480.6>709.4)
Peptide peak area
6.0E+04
5.0E+04
4.0E+04
3.0E+04
2.0E+04
1.0E+04
0.0E+00
0
20
40
60
80
100
120
Egg protein concentration (mg/kg)
× Chocolate ● Cookie ■ Ice cream ▲ Sauce


EALQPIHDLADEAISR (593.3>668.8)
Peptide peak area
2.5E+05
2.0E+05
1.5E+05
1.0E+05
5.0E+04
0.0E+00
0
20
40
60
80
100
120
Egg protein concentration (mg/kg)
× Chocolate ● Cookie ■ Ice cream ▲ Sauce


NIPFAEYPTYK (671.8>558.3)
Peptide peak area
2.5E+05
2.0E+05
1.5E+05
1.0E+05
5.0E+04
0.0E+00
0
20
40
60
80
100
120
Egg protein concentration (mg/kg)
× Chocolate ● Cookie ■ Ice cream ▲ Sauce

Complementary table 1: Blast and homology of selected allergen peptides.

Allergen	Protein	Peptide	Homology (100%)
Milk	Casein αS1 P02662 Bos d 8	FFVAPFPEVFGK	*Bubalus bubalis, Bos mutus*
		YLGYLEQLLR	*Capra hircus, Bubalus bubalis, Ovis aries, Bos mutus*
	Casein αS2 P02663	NAVPITPTLNR	*Bubalus bubalis, Bos mutus*
	P0β-lactoglobulin P02754 Bos d 5	VYVEELKPTPEGDLEILLQK	*Bos mutus grunniens, Bos mutus, Bubalus bubalis*
		VLVLDTDYK	*Bos indicus, Bubalus bubalis, Capra hircus, Bos mutus grunniens, Rangifer tarandus tarandus*
		LSFNPTQLEEQC[+57]HI	*Bubalus bubalis*
Egg	Ovalbumin P01012 Gal d 2	GGLEPINFQTAADQAR	*Achromobacter denitrificans*
		LTEWTSSNVMEER	
		ISQAVHAAHAEINEAGR	*Achromobacter denitrificans, Coturnix coturnix japonica*
	Vitellogenin-2 P02845	EALQPIHDLADEAISR	*Coturnix coturnix japonica, Anolis carolinensis, Meleagris gallopavo, Haliaeetus albicilla, Phaethon lepturus, Balearica regulorum gibbericeps, Merops*
		NIPFAEYPTYK	*Meleagris gallopavo, Anas platyrhynchos, Cuculus canorus, Haliaeetus albicilla, (Balearica regulorum gibbericeps, Phalacrocorax carbo, Chlamydotis*
		NIGELGVEK	*Anas platyrhynchos, Phaethon lepturus, Phalacrocorax carbo, Chlamydotis macqueenii, Merops nubicus, Larus argentatus, Charadrius vociferus, Pelecanus crispus,*
Almond	Prunin Q43607 Pru du 6	GNLDFVQPPR	Prunus persica
		ALPDEVLANAYQISR	
		YNRQETIALSSSQQR	
		QETIALSSSQQR	

CHAPTER IV

DEVELOPMENT OF A STRATEGY FOR THE QUANTIFICATION OF FOOD ALLERGENS IN SEVERAL FOOD PRODUCTS BY MASS SPECTROMETRY IN A ROUTINE LABORATORY

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Context

In a routine laboratory, the detection of peptides from allergens by UHPLC-MS/MS must be accurate and reproducible. Before undertaking the work described in manuscript 4, we had published a method for detecting peptides from ten allergens in processed or complex food products (Planque et al., 2017 a). This method was slightly modified by decreasing the number of peptides per allergen and by keeping two transitions per peptide, allowing the analysis of 10 allergens (milk, egg, soy, peanut, hazelnut, pistachio, cashew, almond, walnut, and pecan nut) in a single analytical run of 26 min within a day.

The sensitivity of the method has been determined on four processed or complex food products, but hundreds of foodstuffs have to be tested for the presence of allergens. Eight target matrices were therefore selected for their high fat (sauce, mayonnaise), protein (ham), carbohydrate (cookie, chocolate, jam, and compote), tannin (spices and chocolate), or polyphenol (ham and compote) content. The eight matrices were spiked with the ten allergens in order to assess allergen detection at the LOQ. On the basis of the results, the rates of false positives and negatives were determined on the basis of a set of criteria such as the retention time, the signal to noise ratio or the tolerance on the ion ratio. These results can provide a basis of reflection and of advices to laboratories regarding the development of a routine UHPLC-MS/MS method for the detection of allergens.

Allergen quantification is a complex task because there exist so many food products. It can be performed using protein extracts or synthetic peptides as standards and either without (Montowska et al., 2017; Pilolli et al., 2018) or with correction with internal standards such as labeled peptides/proteins (Brun et al., 2007; Croote et al., 2017). Depending on the quantification strategy, calibration curves can be established for fortified or incurred matrices, protein extracts, synthetic peptides, or labeled peptides. This usually involves matrix matching with the food product.

In a previous publication (Planque et al., 2017 b), we have shown that the extraction/digestion steps were not fully corrected by adding isotope-labeled peptides: TANELNLLIL[$^{13}C_6{}^{15}N$]R, FFVAPFPEVFGk[$^{13}C_6{}^{15}N_2$], GGLEPINF[D_5]QTAADQAR, EAFGV[D_8]NMQIVR (IS1) as internal standards. In this study and the following manuscript, we introduced new internal standards (long isotope-labeled peptides). Labeled peptides of milk and egg were combined, as were labeled peptides of soy and peanut: G**R**FFV[$^{13}C_5{}^{15}N$]APFPEVFG**K**GGL[$^{13}C_6{}^{15}N$]EPINFQTAADQA**R**GS (milk-egg), and G**R**EAFG V[$^{13}C_5{}^{15}N$]NMQIV**R**TANEL[$^{13}C_6{}^{15}N$]NLLIL**R**GS (soy-peanut) (IS2). The long isotope-labeled peptides must be digested similarly to the protein and should correct at least the digestion step. The main goal being to use a single calibration curve for all the samples that are analyzed. Unfortunately, long isotope-labeled peptides (IS2) did not allow any improvement for the correction of the extraction/digestion steps.

A second strategy for the quantification of proteins was also tested, due to the impossibility of using a single calibration curve for the quantification of food allergens, combining labeled internal standards IS1 and IS2 and standard addition. This strategy consists in adding to the sample increasing amounts of allergens and a fixed amount of labeled internal standard in order to draw a calibration curve and determine the initial concentration of the target allergen that can be detected. Allergen quantification was tested on both spiked matrices and incurred chocolate dessert obtained from Prof. Clare Mills of the University of Manchester.

The recovery of milk, egg, soy, and peanut allergen proteins from the eight food matrices must meet specification of the standard method performance requirements (SMPR 2016.002) for detection and quantitation of selected food allergens that needs to be comprised between 60 -120%.

Major achievements

The second strategy combining a labeled internal standard (IS1 and IS2) with standard addition proved promising. The recoveries determined for milk, egg, soy, and peanut allergens at two concentrations (LOQ and 10 x LOQ) in eight matrices met SMPR specification [60 -120%] in 81.7% and 83.3% of cases after internal standard correction with labeled IS1 and long labeled IS2 peptides, respectively. The second strategy was also used to quantify peanut proteins in an incurred chocolate matrix containing 0, 2, 4, 10, or 30 mg peanut proteins per kg. In this experiment, the recovery of peanut proteins ranged from 50 to 93% after labeled internal standard correction IS1 and from 47% to 87% with long labeled internal standard correction IS2. The recoveries obtained after IS1 and IS2 correction were different and these differences were most likely due to the difference of extraction yield between the labeled internal standards due to matrix effect and the difficulty of digestion for long isotopically labelled peptides. The strategy combining standard addition with labeled peptides thus allows quantifying allergens in all kinds of foodstuffs and this, with a good recovery.

Abstract

Over the last few years, important improvements have been made in the detection of allergens by Mass spectrometry is widely used for detecting and quantifying allergens in foodstuffs. The criteria used to declare a sample positive or negative tend to diverge between laboratories and must be carefully set. In this study, several criteria are highlighted and discussed with a view to avoiding as many false positives and false negatives as possible. Two strategies were tested for the quantification of milk, soy, peanut, and egg allergens in eight food products characterized by very different chemical properties. The first strategy, using labeled and long labeled peptides, was tested with a view to using a single calibration curve. The second strategy involved the combined use of labeled peptides and standard addition of the target allergen. Peanut proteins were quantified in incurred chocolate desserts and recoveries ranging from 50% to 93% with labeled peptide correction were obtained, showing the capacity of the method to quantify allergens in incurred food products.

Keywords

UHPLC-MS/MS, multi-allergens, detection, quantification strategies, labeled peptides, standard addition.

- Quantification strategy: standard addition and labeled peptides
- A simple UHPLC-MS/MS allergen method for routine laboratories
- Sensitive detection of 10 allergens by UHPLC-MS/MS in complex foodstuffs
- Determination of recoveries for spiked and incurred samples
- Analysis and quantification of allergens in incurred and processed samples

1 Introduction

Food allergies are pathological disorders of the immune system, affecting 5% of adults and at least 8% of children in western countries (Sicherer et al., 2014). After an adverse reaction, the allergic population must strictly avoid the offending food. Consequently, food labeling must be clear and reliable (Taylor et al., 2006). In Europe, the relevant legislative texts (regulation 2011/1169/EC) require declaring 14 allergens (and products thereof) on food labels when they are incorporated as ingredients (milk, eggs, cereals containing gluten (wheat, rye, and barley), fish, crustaceans, mollusks, tree nuts (almonds, hazelnuts, walnuts, cashews, pecan nuts, Brazil nuts, pistachio, macadamia), soy, peanuts, sesame, lupine, mustard, celery, and sulfur dioxide (sulfites) (Regulation No. 1169/2011, 2011). Yet the presence of hidden allergens, due to cross-contamination during food production, and the lack of legal action thresholds have encouraged industrialists towards excessive use of precautionary allergen labeling (PAL), i.e. the use of statements such as “may contain…” The unregulated and extensive use of PAL has led to loss of consumer confidence in labeling (Pele et al., 2007; Allen et al., 2014 b; DunnGalvin et al., 2015). Recently, legal thresholds have been set by various countries, such as Switzerland (Crevel et al., 2008), Germany (Waiblinger et al., 2017), Belgium (Federal Food Chain Safety Authority), and the Netherlands (Netherlands Food and Consumer Product Safety Authority). Yet major variations in legal thresholds are reported between European countries, such as 0.0043 mg egg proteins per kilogram in the Netherlands to 1 g egg proteins per kg in Switzerland (ingredients or contaminants). Furthermore, allergens are analytically undetectable at the legal thresholds set in the Netherlands, while those set by Switzerland could endanger the allergic population. In Australia and New Zealand, the Voluntary Incidental Trace Allergen Labeling (VITAL) system establishes eliciting doses (EDs) based on clinical studies for the protection of at least 95% (ED05) of allergic people (Allen et al., 2014 a; Taylor et al., 2014 b). VITAL thresholds, which have no regulatory status, are set at 0.75 mg per kg for egg proteins, 2.5 mg per kg for milk or tree nut proteins, 5 mg per kg for peanut proteins, 25 mg per kg for soybean proteins, and 50 mg per kg for cashew proteins (portion size: 40 g).

Several methods have been developed for the sensitive detection of several allergens in processed or unprocessed matrices (Heick et al., 2011; Gomaa et al., 2015; Korte et al., 2016; Planque et al., 2017 a; Pilolli et al., 2018). Per kg incurred chocolate, for example, limits of quantification of 0.2 to 0.4 mg for milk, 1.0 to 4.0 mg for soy, 2.5 to 4 mg for peanut, and 1 to 3 mg for tree nuts have been obtained (Gu et al., 2018). In another study, egg white, skimmed milk, peanut, soy, and tree nuts (almond, Brazil nut, cashew, hazelnut, pecan, pine nut, pistachio, and walnut) were detected with a limit of detection (LOD) at 10 mg ingredient per kg incurred bread (40 min -180°C) or cookies (18 min -180°C) (New et al., 2018). Boo *et al*. detected down to 5 mg egg, milk, or peanut ingredients per kg incurred

sugar cookie (25 min - 190°C) (Boo et al., 2018). In processed cookies, tomato sauce (45 min - 95°C), chocolate, and ice cream, we were able to detect target allergens at 0.5 mg/kg for milk proteins, 2.5 mg/kg for peanut, hazelnut, pistachio, and cashew proteins, 3 mg/kg for egg proteins, and 5 mg/kg for soy, almond, walnut, and pecan proteins (18 min -180°C) (Planque et al., 2017 a). Although there is still room for improvement in allergen detection, the current challenge is allergen quantification.

An AOAC guideline (SMPR 2016.002) entitled "Standard Method Performance Requirements for Detection and Quantitation of Selected Food Allergens" has been published for the detection and quantification of allergens by mass spectrometry (MS). This guideline sets a recovery of [60-120%] and a relative standard deviation (RSD) of 20%, to be respected by MS methods (Paez et al., 2016). In mass spectrometry, two main strategies are used to quantify allergens in food matrices, and the quantification results obtained with these strategies must be compared with the specifications laid down in the AOAC guideline (Planque et al., 2017 c).

The first strategy avoids using labeled compounds. The concentration of the target allergen in the sample is determined with an external calibration curve (usually a matrix-matched calibration curve) or by standard addition, which consists in adding known amounts of a standard solution of the target allergen directly to the sample in order to draw a calibration curve and determine its initial concentration (Planque et al., 2017 c). For example, Pilolli *et al.* used label-free quantification and compared the peak areas of milk, egg, soy, peanut, and hazelnut marker peptides in fortified cookies (raw ingredients added to the matrix after the process) and spiked cookie extracts (mixed allergen solution added to the matrix). Fortification/spiking was done at 300 and 600 mg ingredients per kg cookies). Recoveries from 51 to 95% was achieved for the five allergens (Pilolli et al., 2018). Despite the lack of thermal processing, the use of the same food matrix, and the high concentration of allergens in the sample, the recoveries achieved by this method did not totally meet the AOAC specifications.

The second strategy is based on labeled peptide or protein quantification. It consists in adding labeled peptides or proteins as internal standards at different stages of the protocol (prior to extraction, digestion, purification, or injection) in order to correct the peak area corresponding to the unlabeled (light) peptide with that corresponding to the labeled (heavy) peptide (Brun et al., 2007). The introduction of heavy peptides will allow correcting for matrix effects and for some protocol steps, depending on the labeled internal standard used (Planque et al., 2017 b). The labeled peptide strategy is increasingly used to quantify allergens by mass spectrometry (Croote et al., 2017; Boo et al., 2018; Groves et al., 2018; Sayers et al., 2018). Boo *et al*., for example, after ensuring that their method was able to detect processed allergens with high sensitivity, quantified milk, egg, and peanut allergens by LC-MS/MS in cookies fortified after the thermal process. After peptide peak area correction with the help of the corresponding labeled peptides, the concentration was determined

with a matrix-matched calibration curve constructed using light and heavy synthetic peptides. The mean recovery was 77 ± 20% (Boo et al., 2018). Huschek *et al*. also used labeled peptides with an external calibration curve constructed with labeled peptides in wheat. For soy, lupine, and sesame allergens, they obtained recoveries from 70 to 113% for cookie, bread, and wheat samples spiked after the thermal process (Huschek et al., 2016). Despite the use of labeled internal standards, the AOAC specifications were not always met, and one should note that the matrices used were of a single type. This strategy was also used to quantify peanut in an incurred chocolate dessert matrix. Using a calibration curve constructed with labeled peptides, the investigators achieved recoveries of 28 to 43%. This just proves that AOAC specifications cannot be applied for the quantification of allergens in commercial samples (Sayers et al., 2018). The main disadvantage of isotope labeled peptides is the lack of correction for losses due to incomplete protein extraction or digestion (Croote et al., 2016). To improve the allergen quantification, two main types of labelled internal standard have been tested in the literature. The first is long isotope-labeled peptides, in which several labeled peptides are linked together or, in which few amino acids are added at each end of the target labeled peptide and the second is the use of labeled proteins. This strategy requires digestion of the labeled internal standard or of the labeled proteins. The prohibitive cost of labeled proteins limits their use, but It is often considered a "gold standard" in the literature (Brun et al., 2007; Ma et al., 2017).

Chen *et al.* compared the use of the isotope-labeled peptide VL[$^{13}C_6$,^{15}N]PV[$^{13}C_5$,^{15}N]PQK with that of the long isotope-labeled peptide QSVLSLSQSKVL[$^{13}C_6$,^{15}N]PV[$^{13}C_5$,^{15}N]PQKAVPYPQRQ. The long isotope-labeled peptide allowed better recovery, thanks to correction for digestion-step-related effects. For cookies spiked after the thermal process, the recovery was between 98.8 and 106.7% (Chen et al., 2015 b). This strategy was tested on a single allergen in a single matrix, but as the recovery was promising, this could be a strategy for avoiding the use of labeled proteins. Newsome *et al.* quantified allergenic bovine milk α_{S1}-casein in processed cookies (180°C-16min) using a recombinant ^{15}N-αS1-casein protein (purity >85%) as internal standard. They obtained a recovery between 60 and 80%, but did not specify that obtained with spiked samples (Newsome et al., 2013).

We have previously developed a sensitive method for detecting ten allergens (egg, milk, soy, peanut, almond, cashew, walnut, pecan nuts, hazelnut and pistachio) in complex and processed matrices (ice cream, sauce (95°C – 45 min), cookie (180°C – 18 min), and chocolate) (Planque et al., 2016). Here, with the aim to improve the routine qualitative method, we have developed a single-run method allowing the analysis of the 10 target allergens within a day. Confirmation criteria, notably regarding the signal to noise ratio (S/N), the tolerance on the retention time, and the tolerance on the relative ion intensity for allergen detection, were tested and set to limit the number of false positives and negatives. Two quantification strategies were tested and compared: (1) the use of a single calibration curve to quantify allergens in several food products, using labeled peptides and long labeled peptides

(which appeared promising for the detection of milk in cookies in the study of Chen *et al*. (Chen et al., 2015 b)), (2) a combination of standard addition with the use of a labeled peptide as internal standard, which constitutes the originality of this work. As proof of concept, this second strategy was tested on eight spiked matrices and on incurred chocolate dessert matrices to provide recovery results for "real samples". To the best of your knowledge, combining standard addition with a labeled internal standard strategy has never been done for the quantification of allergens in several food products. This strategy allows quantifying several allergens in several food products belonging to different food categories. It is thus totally suitable for allergen quantification in routine laboratories.

2 Materials and Methods

2.1 Materials and reagents

Tris(hydroxymethyl)aminomethane (Tris), urea, dimethyl sulfoxide (DMSO), DL-dithiothreitol (DTT), iodoacetamide (IAA), ammonium bicarbonate, and trypsin from bovine pancreas (T8802) were obtained from Sigma-Aldrich (Bornem, Belgium). Acetic acid was obtained from Acros Organics (Geel, Belgium) and hydrochloric acid was from Fisher Chemical (Loughborough, UK). Sep-Pak C18 solid phase extraction (SPE) columns (6 cc, 500 mg - WAT043395) were used for peptide purification and enrichment and purchased from Waters (Milford, Massachusetts, USA). Acetonitrile, 2-propanol, methanol (ULC-MS grade), waters, hexane, and formic acid were from Biosolve (Valkenswaard, the Netherlands). The labeled peptides TANELNLLI**L** [$^{13}C_6{}^{15}N$] R, FFVAPFPEVFG**K** [$^{13}C_6{}^{15}N_2$], GGLEPIN**F** [D_5] QTAADQAR, EAFG**V** [D_8] NMQIVR, GRFF**V**[$^{13}C_5{}^{15}N$]APFPEVFGKGG**L**[$^{13}C_6{}^{15}N$]EPINFQTAADQARGS, and GREAFG**V**[$^{13}C_5$ ^{15}N]NMQIVRTANE**L**[$^{13}C_6$ ^{15}N]NLLILRGS were from Eurogentec (Seraing, Belgium). Milk powder (NIST1549a 25.64% protein), soy flour (NIST 3234 53.37% protein), peanut butter (NIST 2387 22.2% protein) and whole egg (NIST 8445 48% protein) were obtained from the National Institute of Standards and Technology (NIST) (Gaithersburg, Maryland, USA). Tree nuts (almonds, cashews, pecan nuts, hazelnuts, walnuts, and pistachios) were purchased from a local store before being finely ground under liquid nitrogen. An Acquity liquid chromatograph coupled wiith a Xevo TQS triple quadrupole system (Waters - Milford, Massachusetts, USA) was used with a C18 Acquity BEH130 column (Waters - 2.1 x 150 mm – ref. 186003556).

2.2 Composition of target matrices

Eight target matrices were selected. Six were purchased from a local store (chocolate, compote, jam, chicken ham, Andalusian sauce and smoked paprika spices) and two were home-made (cookie and mayonnaise). Chocolate (containing 45% cacao, 35% sugar, and 20% rice powder), apple and pear compote (64.9% apple and 35% pear), jam (65% wood fruit and 33% sugar), Andalusian sauce (oil,

15% tomato, egg yolk, glucose syrup, vinegar, 3.5% mustard, sugar, lemon juice, salt, spices, and may contain milk), chicken ham, and spice (smoked paprika) were from the local store. Cookie dough was prepared by mixing 53.4% flour, 15.2% sugar, 16.1% oil, 14.8% water, 0.3% salt, 0.1% ammonium bisulfate, and 0.1% sodium bicarbonate with a blender. Cookies (40 g each) were finally cooked at 180°C for 18 min. Mayonnaise was prepared by combining 33.3% oil, 33.3% cornstarch, 23.3% mustard, and 10% vinegar. To ensure homogeneity, the matrices were finely ground before being weighed for analysis.

2.3 Preparation of labeled internal standard solutions

Stock solutions were prepared by dissolving labeled peptides in DMSO (10 mg/mL) before diluting with 0.1% formic acid to obtain a final concentration at 1 mg/mL. Stock solutions were stored at -20°C. Working solution was prepared in 0.1% formic acid with the stock solutions of FFVAPFPEVFGK [$^{13}C_6{}^{15}N_2$] (5.25 µg/mL), EAFGV [D_8] NMQIVR (9.25 µg/mL), TANELNLLI**L** [$^{13}C_6$ ^{15}N] R (9.25 µg/mL), GGLEPIN**F**[D_5]QTAADQAR (9.25 µg/mL), GRFF**V**[$^{13}C_5{}^{15}N$]APFPEVFGKGG**L**[$^{13}C_6{}^{15}N$]EPINFQTAAD QARGS (10 µg/mL), and GREAFG**V**[$^{13}C_5{}^{15}N$]NMQIVRTANE**L**[$^{13}C_6{}^{15}N$]NLLILRGS (10 µg/mL).

2.4 Preparation of the standard protein working solution

On the basis of NIST or VITAL protein contents, a solution was prepared containing the following allergens at the following concentrations (expressed in µg total proteins per mL): 75 µg/mL for milk, 112.5 µg/mL for egg, 750 µg/mL for soy, and 375 µg/mL for peanut and tree nuts. The proteins were extracted with 200 mM Tris-HCl pH 9.2, 2 M urea by shaking at 20°C for 30 min (Agitelec, France) followed by ultrasound treatment at 4°C for 15 min. After centrifugation at 4660 g for 10 min at 4°C, this solution was used to spike samples prior to applying the protocol.

2.5 Extraction, digestion and purification of samples

The protocol described in Planque et *al.* (2017) was used. Briefly, the proteins contained in 3 g sample, previously spiked with 100 µL of labeled internal standard solution, were extracted with 30 mL extraction buffer (200 mM TRIS-HCl pH 9.2, 2 M urea) by shaking at 20°C for 30 min (Agitelec, France) followed by sonication for 15 min at 4°C. After centrifugation at 4660 g for 10 min at 4°C, the proteins contained in 10 mL supernatant were diluted with 10 mL of 200 mM ammonium bicarbonate. Protein reduction and alkylation were performed, respectively, with 1 mL of 200 mM DTT (45 min – 20°C) and 1 mL of 400 mM IAA (45 min – 20°C in the dark). Digestion was achieved by adding tryspin (1 mL of 1 mg trypsin/mL in 50 mM acetic acid) and incubating for 1 h at 37°C. Digestion was stopped by addition of 300 µL of 20% formic acid. Peptides were concentrated and purified on C18 SPE cartridges. Cartridge conditioning was done with 18 mL acetonitrile followed by

18 mL of 0.1% formic acid. After centrifugation of the peptide extract at 4660 g for 10 min at 20°C, 20 mL supernatant was loaded on the column and impurities were flushed out with 18 mL of 0.1% formic acid. DMSO (30 µL) was added to avoid dryness in the collector tube before peptide elution with 6 mL acetonitrile/0.1% formic acid (80/20, v/v). After evaporation under a nitrogen flow in a water bath set at 40°C, the peptides contained in the pellets were dissolved in 0.1% formic acid/acetonitrile (95/5, v/v) (600 µL). The extracts were centrifuged at 4660 g for 5 min at 10°C, transferred to a microtube, and centrifuged again at 11754 g for 5 min at 4°C. The samples were then analyzed by UHPLC-MS/MS in order to detect 10 allergens simultaneously with a single injection.

2.6 Preparation of test samples

Blank matrices (without target allergens) were analyzed in triplicate to check the specificity of the method. Afterwards, for each matrix, six samples called “C1 samples” were spiked at VITAL thresholds or lower (LOQs or LODs determined in a previous study): per kg of food product, 0.5 mg for milk proteins, 0.75 mg for egg proteins, 5 mg for soy proteins, and 2.5 mg for peanut and tree nut proteins. For each matrix, six samples named “C2 samples” were spiked at a concentration ten times as high as the corresponding C1 level: per kg of food product, 5 mg for milk proteins, 7.5 mg for egg proteins, 50 mg for soy proteins, and 25 mg for peanut and tree nut proteins. The compote matrix was spiked eighteen times at the C2 level in order to determine the relative standard deviation (RSD) of the concentrations of milk, egg, peanut, and soy proteins obtained experimentally with the three replicates. To determine the recovery, standard addition was performed on the six samples at 0, 0.5, 1, 2.5, 5, and 10 mg/kg for milk proteins, 0, 0.75, 1.5, 3.75, 7.5, and 15 mg/kg for egg proteins, 0, 5, 10, 25, 50, and 100 mg/kg for soy proteins, and 0, 2.5, 5, 12.5, 25, and 50 mg/kg for peanut and tree nut proteins.

2.7 Analysis of incurred iFAAM samples

Chocolate dessert matrices were produced by the University of Manchester in the framework of the “Integrated Approaches to Food Allergen and Allergy Management” (iFAAM) project. Chocolate matrices containing 0, 2, 4, 10, and 30 mg peanut proteins per kg were quantified with the new strategy.

2.8 UHPLC-MS/MS parameters for peptide analysis

Peptide separation was performed with an Acquity system (Waters - Milford, Massachusetts, USA) on a C18 Acquity BEH130 Waters column (2.1 x 150 mm) at 50°C at 0.2 mL/min. Peptide elution was carried out for 26 min as follows: 0–3 min: 92% A; 3–18 min: 92% to 58% A, 18.0–18.1 min: 58% to 15% A; 18.1–22.5 min: 15% A; 22.5–22.6 min: 15% to 92% A, 22.6–26 min: 92% A (solvent A: 0.1%

formic acid – solvent B: acetonitrile plus 0.1% formic acid). After sample analysis, the UPLC column was flushed with methanol/2-propanol/acetonitrile/water (25/25/25/25 v/v/v/v) for 15 min before returning to the initial conditions (92% solvent A) for 5 min in order to avoid carry-over (peaks attributed to the previously analyzed sample may be observed in subsequent chromatograms). MRM detection in positive electrospray mode was performed with a Waters Xevo TQS triple quadrupole system. The cone nitrogen flow was set at 150 L/h, the collision gas flow at 0.12 mL/min, the capillary voltage at 2.0 kV, and the source temperature at 150°C. The desolvation temperature was set at 500°C and the nitrogen flow at 1200 L/h.

3 Results and discussion

3.1 Selection of marker peptides

The sensitivity of the method was determined in a previous study for milk (casein and whey), egg (white and yolk), peanut, soy, and tree nuts (almond, hazelnut, walnut, pecan nuts, cashew and pistachio) in processed and complex food products (cookie (180°C-18 min), sauce (95°C-45 min), chocolate (tannin) and ice cream (fat)) (Planque et al. 2017). On the basis of sensitivity and specificity (assessed by BLAST analyses and allergen-free matrix MS analyses), 3 to 5 peptides per allergen, with 3 transitions per peptide, were retained. The limit of quantification (LOQ) of each target allergen was determined on incurred food matrices containing 0.5 mg/kg (casein) and 5 mg/kg (whey), 2.5 mg/kg for peanut, hazelnut, pistachio, and cashew proteins, 3 mg/kg (white egg) and 60 mg/kg (yolk egg) for egg proteins, and 5 mg/kg for soy, almond, walnut, and pecan proteins, considering a signal-to-noise ratio above 10 (Planque et al. 2017; Planque et al. 2016).

Two to five abundant peptides per allergen, with two MRM transitions per peptide, were retained, to allow simultaneous analysis of 10 allergens in a single UHPLC-MS/MS method (**Table 1**). The ability of the method to reach the previously determined sensitivity was checked by analyzing the target allergens at their LOQs in the four processed and complex matrices (data not shown).

***Table 1**: Multiple Reaction Monitoring (MRM) parameters for the identification of milk, egg, soybean, peanut, and tree nut (walnut, pecan nuts, almond, cashew, hazelnut, and pistachio) proteins by UHPLC-MS/MS. The cone voltage was set at 35 V. The sensitivity reached for each peptide in processed and complex matrices (cookie (180°C - 18 min), tomato sauce (95°C – 45 min), chocolate and banana ice cream) has also been specified (M. Planque et al. 2017).*

Allergen	Protein	Peptide	LOQ (mg of proteins per kg) (Planque *et al.* 2017)	Precursor (charge state) (m/z)	Product ion (fragments)	Collision energy (eV)
Soy	Glycinin G2 P04405 Gly m6	EAFGVNMQIVR	5	632.3 (++)	760.4 (y6)	17
					646.4 (y5)	22
	2S albumin P19594 Gly m 2S albumin	ELINLATMC[+57]R	5	610.8 (++)	865.4 (y7)	21
					638.3 (y5)	17
	Glycinin G1 P04776 Gly m6	VFDGELQEGR	10	575.3 (++)	788.4 (y7)	20
					602.3 (y5)	20
Milk	Casein αS1 P02662 Bos d 8	FFVAPFPEVFGK	0.5	692.9 (++)	991.5 (y9)	18
					920.5 (y8)	18
		YLGYLEQLLR	0.5	634.4 (++)	934.5 (y7)	21
					771.5 (y6)	20
	Casein αS2 P02663	NAVPITPTLNR	2.5	598.3 (++)	911.5 (y8)	17
					285.2 (b3)	12
	P0β-lactoglobulin P02754 Bos d 5	VLVLDTDYK	10	533.3 (++)	853.4 (y7)	15
					754.4 (y6)	14
		LSFNPTQLEEQC[+57]HI	5	858.4 (++)	1254.6 (y10)	26
					627.8 (y10)	27
Egg	Ovalbumin P01012 Gal d 2	GGLEPINFQTAADQAR	3	844.4 (++)	1121.5 (y10)	28
					666.3 (y12)	25
		LTEWTSSNVMEER	15	791.4 (++)	1052.5 (y9)	31
					951.4 (y8)	23
	Vitellogenin-2 P02845	EALQPIHDLADEAISR	60	593.3 (+++)	761.4 (y7)	19
					668.8 (y12)	15
		NIPFAEYPTYK	60	671.8 (++)	1115.5 (y9)	15
					558.3 (y9)	29
	Vitellogenin-1 P87498	YLLDLLPAAASHR	60	480.6 (+++)	709.4 (y7)	15
					582.3 (y11)	10
Peanut	Cupin Q8LKN1 Ara h 3/4	RPFYSNAPQEIFIQQGR	5	684.4 (+++)	748.4 (y6)	20
					836.4 (b7)	17
	Cupin Q647H4 Ahy-1	TANELNLLILR	2.5	635.4 (++)	854.6 (y7)	20
					741.5 (y6)	22
	Conglutin 7 Q6PSU2 Ara h2	NLPQQC[+57]GLR	5	543.3 (++)	858.4 (y7)	13
					429.7 (y7)	16
Walnut	Vicilin-like protein Q9SEW4	ATLTLVSQETR	12.5	609.8 (++)	620.3 (y5)	19
					832.5 (y7)	21
	Albumin seed storage protein P93198 Jug r 1	GEEMEEMVQSAR	5	698.3 (++)	949.4 (y8)	22
					820.4 (y7)	22
Pecan nuts	7S vicilin B3STU4 Car i 2	ATLTFVSQER	12.5	576.3 (++)	765.4 (y6)	16
					618.3 (y5)	18
		NFLAGQNNIINQLER	12.5	582.0 (+++)	659.4 (y5)	19
					772.4 (y6)	18
		LVGFGINGK	5	452.8 (++)	692.4 (y7)	12
					488.3 (y5)	13
Almond	Prunin Q43607 Pru du 6	GNLDFVQPPR	5	571.8 (++)	743.4 (y6)	19
					596.4 (y5)	14
		ALPDEVLANAYQISR	5	830.4 (++)	1035.6 (y9)	30
					922.5 (y8)	32
		QETIALSSSQQR	25	674.3 (++)	876.5 (y8)	26
					692.3 (y6)	27
	Pru2 protein Q43608 Pru du 6	TDENGFTNTLAGR	25	698.3 (++)	879.5 (y8)	25
					732.4 (y7)	23
Cashew	Allergen Ana o 2 Q8GZP6 Ana o 2	C[+57]AGVALVR	2.5	423.2 (++)	614.4 (y6)	13
					458.3 (y4)	14
		AMTSPLAGR	2.5	452.2 (++)	701.4 (y7)	13
					513.3 (y5)	15
	2S albumin Q8H2B8 Ana o 3	ELYETASELPR	2.5	654.3 (++)	773.4 (y7)	22
					672.4 (y6)	20
Hazenut	11S globulin- like protein Q8W1C2 Cor a 9	ADIYTEQVGR	5	576.3 (++)	689.4 (y6)	19
					588.3 (y5)	16
		QGQVLTIPQNFAVAK	5	807.5 (++)	1088.6 (y10)	27
					874.5 (y8)	23
		ALPDDVLANAFQISR	2.5	815.4 (++)	1019.6 (y9)	28
					906.5 (y8)	31
Pistachio	11S globulin precusor B7SLJ1 Pis v 5	ITSLNSLNLPILK	2.5	713.4 (++)	1011.6 (y9)	21
					470.3 (y4)	22
		AMISPLAGSTSVLR	2.5	701.9 (++)	1000.6 (y10)	23
					790.4 (y8)	28
	11 S globulin B2KN55 Pis v 2	VTSINALNLPILR	2.5	712.4 (++)	838.6 (y7)	22
					1023.6 (b10)	23
	11S globulin precusor B7P073 Pis v 2	ALPLDVIK	2.5	434.8 (++)	684.4 (y6)	10
					342.7 (y6)	11

In the previous studies, the main goal was to detect allergens in incurred and processed matrices high in carbohydrate. Here it was to determine (1) the capacity of the method to detect allergens in different categories of food matrices (meat, salad dressing, spices…) and (2) the advantage of using labeled peptides for allergen quantification.

Most studies focus on detecting allergens in matrices containing a high percentage of carbohydrates (Planque et al., 2017 c), while only a few have been tested on high-fat matrices such as poultry meat products (sausage or pâtés (25-30% fat)) (Montowska et al., 2018). Routine methods must be able to detect the allergens in all kinds of foodstuffs, such as products with a high content in fat (e.g. mayonnaise, sauce), proteins (e.g. meat, fish), or even both. Eight target matrices were selected on the basis of the percentage of fat, carbohydrate, or proteins (based on the Association of Official Agricultural Chemists (AOAC) triangle (Phillips et al., 2013)). Matrices with a high content in polyphenols (ham and compote) and tannins (spices and chocolate) were also selected to ensure the capacity of the method to detect and quantify allergens in a very wide range of foodstuffs.

The eight food matrices were spiked (addition of extracted allergens after the thermal process) in order to avoid variation factors, such as the degradation of proteins by the thermal process. Indeed, while sensitivity was estimated on processed and incurred samples, the quantification must be done with spiked materials in order to validate the approach (Planque et al., 2017 a)**.**

3.2 Determination of the sensitivity

For the 2 to 5 selected peptides per allergen, the two MRM transitions in allergen-free and LOQ-contaminated matrices were presented to demonstrate the specificity and sensitivity of the method in the eight target matrices (**Supplementary material – Figure 1**). This analysis revealed that the sauce is containing egg and milk allergens, originating either as an ingredient or from cross-contamination (may contain labeling) in the preparation. Consequently, the sensitivity and the specificity of milk and egg peptide detection could not be determined in this matrix. The choice of the matrices was based on two criteria: a high percentage of fat, carbohydrate or proteins and the samples that are the most commonly analyzed in routine laboratories (**Figure 1**).

The ten allergens have been analyzed in the eight target matrices by scanning two transitions for 35 selected peptides. The LOQs obtained in spiked samples were compared with the LOQs previously determined on the four incurred and processed food products as reported in the table 1 (Planque et al., 2017 a). Although a lower sensitivity in processed and incurred matrices could have been expected due to the impact of process on the protein, the observed sensitivity was revealed higher in some matrices, such as in sauce, mayonnaise and paprika spice for EAFGVNMQIVR soy peptide (**Figure 1**). This phenomenon was observed for 20.7% of the determined LOQs (56 out of 270). Such

increased method sensitivity was mostly associated with fat matrices (mayonnaise (6.3%) and sauce (4.1%)) and spices (6.3%) (**Supplementary material – Figure 2**).

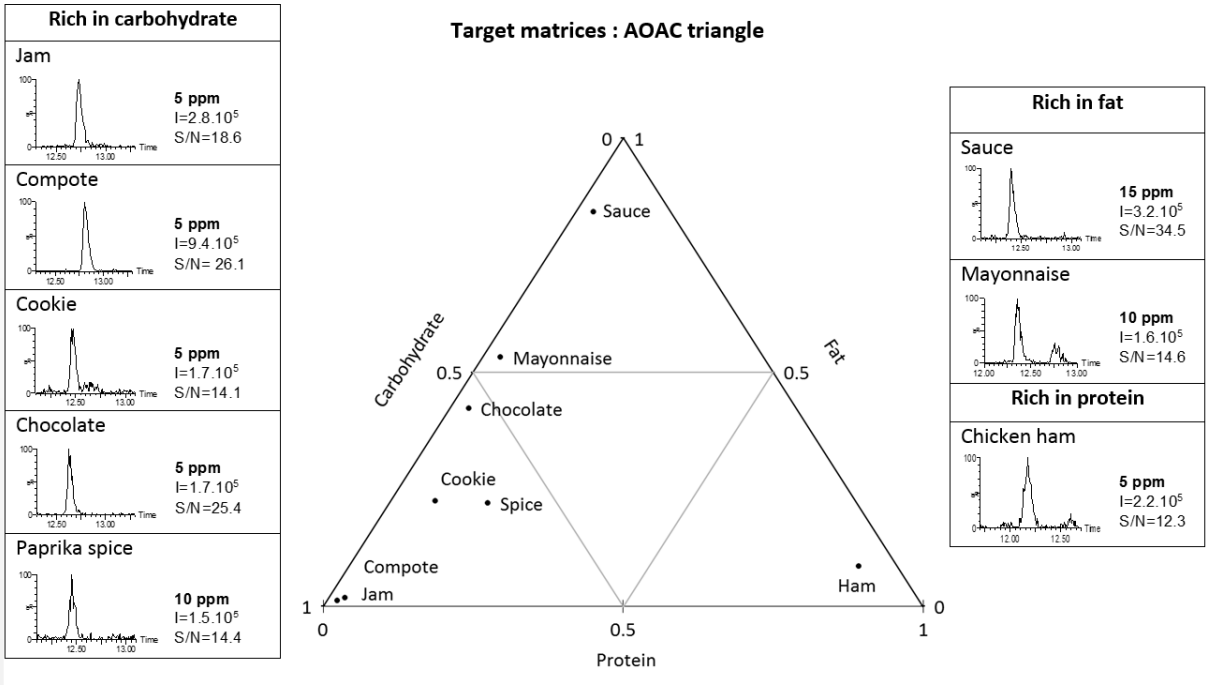


Figure 1: *Chromatograms of EAFGVNMQIVR soy peptide (632.3 > 646.4) spiked at the LOQ (S/N>10) in eight matrices classified in function of the percentage of carbohydrate, protein and fat.*

However, the LOQs of yolk egg peptides were approximately 4 times lower in spiked cookies than in processed cookies (**Supplementary material – Figure 1**). This result indicates that the selection of marker peptides for the detection of allergens and the determination of the method sensitivity should always be done in processed matrices. However, as described above, a standard addition (the target allergen is spiked on the matrix after the process) should also be systematically performed, during the first analysis of a food product, in order to guarantee the detection of the allergens at the LOQ. Moreover differences could also be observed in matrices that could be considered as "identical". For instance, the LODs for GPFPIIV peptide from β-casein has been shown to vary from 0.09 to 0.23 mg/L in five commercial white wines (Losito et al., 2013). By consequence, standard additions should also be done in "similar" food products.

3.3 Acceptance criteria for positive samples

Several criteria must be taken into account in order to declare a sample as positive or negative. The main question, largely debated between laboratories, is to decide if a sample has to be considered as positive with one single detected allergen peptide or if at least two peptides must be detected. To answer this question, different parameters have to be considered such as the signal to noise ratio (S/N), the tolerated retention time deviations, and the relative ion intensity.

We have analyzed our data set composed of the UHPLC-MS/MS analyses of eight complex and/or processed matrices spiked with ten different allergens at six level of concentrations. For milk, egg, soy, peanut, and tree nut proteins, the two most intense or specific transitions per peptide, previously analyzed in the four incurred matrices (processed and complex), were retained in order to analyze ten allergens in a single analytical run (Planque et al., 2017 a). To assess the rate of false positives, blank (i. e. allergen-free) matrices were also analyzed.

3.3.1 The signal to noise ratio

The first parameter considered was the minimum S/N ratio for a positive sample. It is generally accepted in the literature that the S/N ratio must be higher than 3 for a limit of detection and 10 for a limit of quantification for the most intense transition (Peters et al., 2007). The samples were considered positive only if both MRM transitions gave a chromatographic signal at the same retention time with S/N ratios of 10 and 3 for the first and second selected transitions, respectively. In our data set, the LOQs for hazelnut in mayonnaise (5 mg/kg), walnut in ham and smoked paprika spice (7.5 mg/kg), soy in smoked paprika spice (10 mg/kg), and egg in mayonnaise (4.5 mg/kg) (expressed in mg proteins per kg food) were higher than those previously determined for processed and incurred food products.

Considering one positive peptide per allergen, this method generates 5.1% false negatives (4 peptides out of 78 (10 allergens in 8 matrices, excluding egg and milk in sauce)). It is therefore of utmost importance to spike target food matrices at the LOQ to ensure reliable detection of allergens. To consider a sample negative, there should be no signal at the relevant retention time (RT) or the S/N ratios should lower than 10 and 3. Yet defining such S/N ratio criteria immediately raises a second question: what is the tolerance of retention time between the standard and the sample?

3.3.2 Tolerated retention time variation.

The guideline SANTE/11813/2017 for pesticides states that the difference of RT observed for the (matrix-matched)) calibration standard and the sample should be less than or equal to ± 0.1 min (SANTE/11813/2017, 2017). In Regulation 2002/657/EC for the analysis of veterinary drug residues, on the other hand, the tolerance is set at 2.5% for liquid chromatography analyses (Direction 2002/657/EC, 2002).

In our data set, blank matrices displayed two MRM transitions at retention times similar to those of the target peptide in the case of 11 peptides (pistachio: AMISPLAGSTSVLR in sauce, hazelnut: ALPDDVLANAFQISR in ham, almond: QETIALSSSQQR in mayonnaise, pecan nuts: LVGFGINGK in cookie, NFLAGQNNIINQLER in spice and ATLTFVSQER in mayonnaise, milk: LSFNPTQLEEQCHI in spice,

peanut TANELNLLILR in ham and sauce, and RPFYSNAPQEIFIQQGR in ham and sauce) (**Supplementary material – Figure 1**).

We analyzed the effect of applying the two recommended RT tolerance thresholds (± 0.1 min and 2.5%) on the specificity of our detection method, considering detection of each allergen with a single peptide. We calculated the difference in RT between the target peptide at the LOQ and the impurity in the blank. Considering a tolerated RT difference of 2.5% or less, LVGFGINGK (cookie) and TANELNLLILR (sauce) peptides could be excluded from the false positive list. Consequently, considering one positive peptide per allergen, this method generates 10.3% false positives (8 peptides out of 78 (10 allergens in 8 matrices, excluding egg and milk in sauce)). Assuming a (lower) tolerated RT difference of 0.1 min, 4 peptides should be considered positive: NFLAGQNNIINQLER (spice), ATLTFVSQER (mayonnaise), LSFNPTQLEEQCHI (spice), and RPFYSNAPQEIFIQQGR (ham). This translates as a false positive rate of 5.1%. Despite this lower rate of false positives at lower tolerance, we recommend considering a tolerance of 2.5% as for veterinary drug residues (Direction 2002/657/EC, 2002), as RT variations might be observed in similar matrices with an external calibration curve. It is worth stressing, however, that the use of standard addition solves the problem of retention time deviation tolerance, as it enables distinguishing impurities from target peptides, as shown in Figure 1-Supplementary material. Our data show, however, that a doubt can subsist in the case of impurities displaying the same retention time as target peptides: in the depicted experiment, a doubt remained regarding the pecan nut peptide ATLTFVSSQER in mayonnaise and the peanut peptide RPYSNAPQEIFIQQGR in chicken, where the nearly identical retention times of the impurities and target peptides led to a false positive rate of 2.6%.

With the same parameters (10 and 3 S/N ratios; 2.5% RT deviation tolerance), we examined how false positive and false negative rates would be affected by considering two peptides for allergen detection instead of only one. In our data set, TANELNLLILR and RPFYSNAPQEIFIQQGR were detected in allergen-free ham. This means 1 false positive result out of 78, corresponding to a 1.3% false positive rate, much lower than the 10.3% rate obtained when the adopted criterion was 1 positive peptide per allergen. However, 33.3% of positive samples (26 cases out of 78) at a concentration higher than the LOQ previously determined in incurred matrices should be declared as negative if two peptides per allergen must be detected with a signal to noise ratio of 10 and 3 for the first and second selected transitions, respectively (**Supplementary material – Figure 1**). By consequence, the criteria of at least two peptides per allergens to declare a sample as positive should not be applied: although very specific (1.3% of false positive), it is not sensitive enough (33.3% of false negative). Therefore, the criteria of detecting one single peptide looks preferable to two peptides, especially when considering the relative ion intensity of the peptide that can decrease the rate of false positive samples.

3.3.3 Tolerated relative ion intensity deviation

The ion ratio is the intensity ratio between the second and first transitions. After calculation of each ion ratio, that observed for the sample is compared with that observed for the standard, and a "relative ion intensity" is determined, expressing as a percentage the difference between the two ion ratios. One can then compare the calculated relative ion intensity with the maximum permitted tolerance for relative ion intensity. According to guideline SANTE/11813/2017 for pesticide analysis, a peptide will be confidently detected and considered positive if the ion ratio deviation between different allergen concentrations does not exceed 30%. Regulation 2002/657/EC for the analysis of veterinary drug residues adopts the same criterion, with an added nuance: when the ion ratio is low (i.e. the intensity of the second transition is no more than 10% lower than the intensity of the first), a higher ion ratio deviation between allergen concentrations can be tolerated (**Supplementary material – Figure 3**).

In our dataset, the ion ratio deviation between two different concentrations (LOQ and 20 x LOQ) was calculated and compared with the tolerated deviation set by Regulation 2002/657/EC. The choice of the concentration 20 x LOQ was justified by a high matrix effect and the impossibility of using a single standard matrix-matched with all food products. For peanut peptide RPYSNAPQEIFIQQGR, the relative ion intensity deviation observed between the LOQ (from 2.5 to 27.5 mg) and 52.5 mg peanut proteins per kg was 5.9 to 19.6%, except in the case of chicken ham, characterized by a 965.7% relative ion intensity deviation (**Supplementary material – Table 1**). This led us to exclude the RPYSNAPQEIFIQQGR peptide for the determination of positive chicken ham matrix samples. As we know that this matrix was spiked with peanut, our data indicate that using the ion ratio criterion generates false negative results with this peptide, although the samples could be considered positive on the basis of the data for the peanut peptides TANELNLLILR and NLPQQCGLR. However, applying the ion ratio criterion did allow avoiding false positive results when the RPYSNAPQEIFIQQGR peptide was excluded in the detection of peanut in the blank ham matrix.

A similar problem was observed for the ATLTFVSSQER pecan nut peptide. The relative ion intensity deviation between the LOQ (from 2.5 to 27.5 mg) and 52.5 mg pecan nut proteins per kg ranged from 1.3 to 14.7%, except in the case of mayonnaise, for which a 36.8% relative ion intensity deviation was observed. As the ion ratio for ATLTFVSSQER peptide in mayonnaise was higher than 50%, the tolerated ion ratio deviation should not exceed 20% (2002/657/EC) or 30% (SANTE/11813/2017). This made it necessary to exclude this peptide. As the two remaining two false positives observed with blank matrices were excluded on the basis of the relative ion intensity criterion, this criterion should be systematically applied to avoid false positives.

In conclusion, it is possible to avoid false positives by combining the standard addition strategy, signal to noise ratios of 10 and 3 for the first and second transitions, respectively, a 2.5% tolerance for the retention time, and existing criteria regarding the tolerated relative ion intensity deviation.

3.4 Quantification strategy

The SMPR 2016.002 "Standard Method Performance Requirements for Detection and Quantitation of Selected Food Allergens" sets a recovery of [60-120%] and a maximum relative standard deviation (RSD) of 20% for the validation of allergen methods based on mass spectrometry. We thus examined our dataset corresponding to eight matrices used for the quantification of milk, egg, soy, and peanut allergens in the light of these recovery and RSD specifications.

3.4.1 External calibration curve with labeled internal standard correction

The ideal quantification strategy for routine laboratories would involve using a single calibration curve based on measurements in solvent or on a single matrix-matched calibration to quantify allergens in different food samples, no matter the kind of food matrix to be analyzed. This strategy implies careful selection of the internal standard in terms of both specificity and sensitivity, in order to reach optimal method performance. The first tested internal standard (IS 1) in this study contained the following labeled peptides: FFVAPFPEVFGK[$^{13}C_6{}^{15}N_2$] for milk, GGLEPINF[D_5]QTAADQAR for egg, TANELNLLIL[$^{13}C_6{}^{15}N$]R for peanut and EAFGV[D_8]NMQIVR for soy. This standard proved adequate for correcting the matrix effect and effects related to the purification step and UHPLC-MS/MS analysis variability. It did not adequately correct for extraction and tryptic digestion variability, however, although these steps are crucial for method performance (Planque et al., 2017 b).

In the present study, a second internal standard (IS 2) was tested: use of the long labeled peptides GRFF**V**[$^{13}C_5{}^{15}N$]APFPEVFGKGG**L**[$^{13}C_6{}^{15}N$]EPINFQTAADQARGS for milk and egg and GREAFG**V**[$^{13}C_5{}^{15}N$]N MQIVRTANE**L**[$^{13}C_6$ ^{15}N]NLLILRGS for soy and peanut. As long labeled peptides should be digested prior to MS analysis, this should allow correction for digestion step variability.

The IS 1 or IS 2 labeled peptides were introduced before the extraction step, and the eight target matrices were spiked at 6 concentration levels: 0, 0.5, 1, 2.5, 5, and 10 mg/kg for milk proteins, 0, 0.75, 1.5, 3.75, 7.5, and 15 mg/kg for egg proteins, 0, 5, 10, 25, 50, and 100 mg/kg for soy proteins, and 0, 2.5, 5, 12.5, 25, and 50 mg/kg for peanut and tree nut proteins. The peak areas of milk, egg, soy, and peanut peptides in the eight matrices were corrected after digestion on the basis of the area for IS 1 or IS 2 (FF**V**[$^{13}C_5{}^{15}N$]APFPEVFGK (milk) GG**L**[$^{13}C_6{}^{15}N$]EPINFQTAADQAR (egg), EAFG**V**[$^{13}C_5{}^{15}N$]NMQIVR (soy) TANE**L**[$^{13}C_6{}^{15}N$]NLLILR (peanut)). Linear curves obtained after correction with the IS 1 or IS 2 labeled peptides were drawn for milk, egg, soy, and peanut peptides (**Figure 2**).

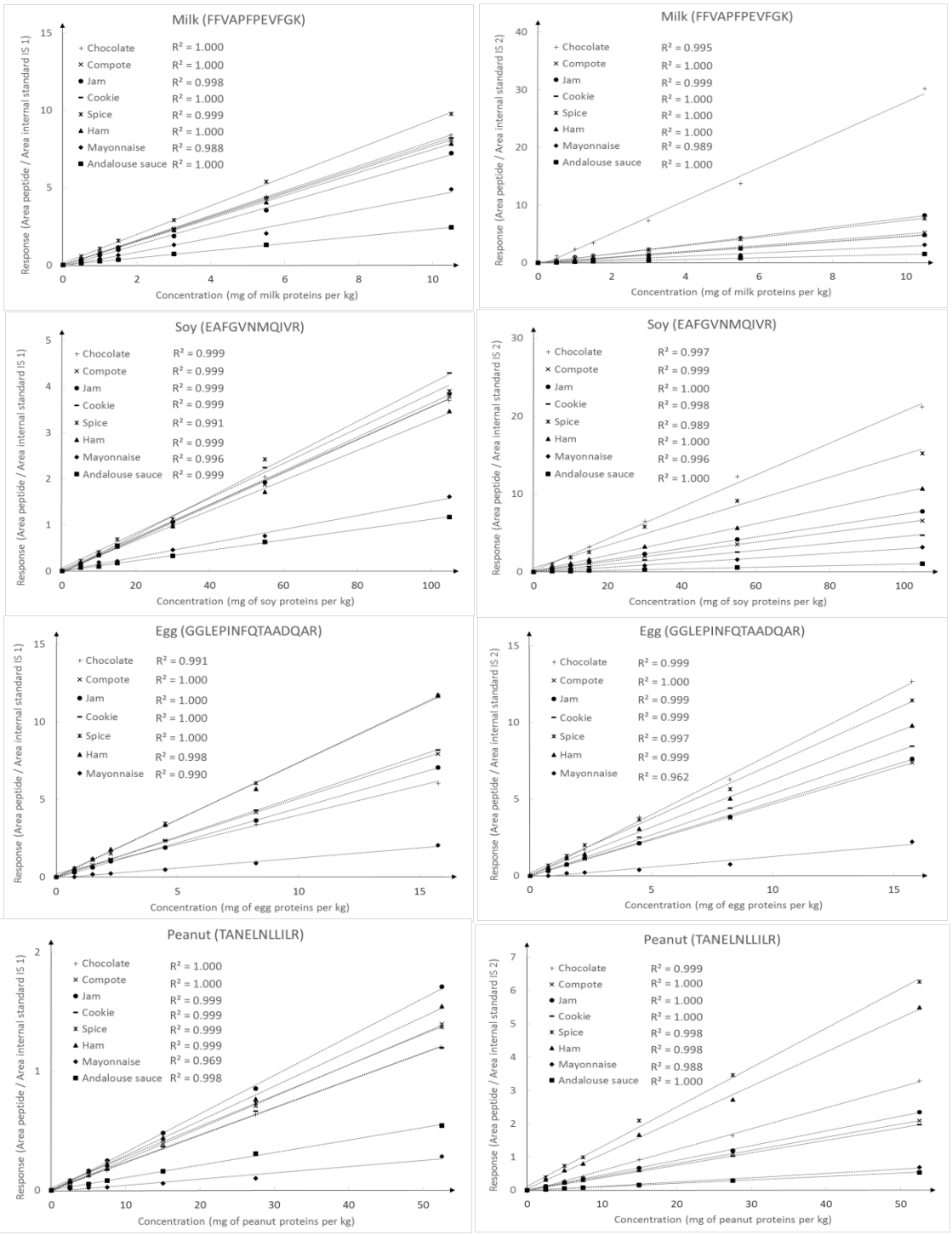


Figure 2: *Linear regression of peptide peak areas corresponding to the highest MRM transition corrected with the labeled IS 1 peptide ((FFVAPFPEVFGK[$^{13}C_6{}^{15}N_2$] (milk), GGLEPINF[D_5]QTAADQAR (egg), TANELNLLIL[$^{13}C_6$ ^{15}N]R (peanut), EAFGV[D_8]NMQIVR (soy)) or the long labeled IS 2 peptide (GRFFV[$^{13}C_5{}^{15}N$]APFPEVFGKGGL[$^{13}C_6{}^{15}N$]EPINFQTAADQARGS (milk and egg) and GREAFGV[$^{13}C_5{}^{15}N$]NM QIVRTANEL[$^{13}C_6{}^{15}N$]NLLILRGS (soy and peanut)) as a function of the concentration of food allergen proteins in spiked chocolate, compote, jam, cookie, spice, ham, and mayonnaise. The linearity was checked for milk casein FFVAPFPEVFGK (692.9 > 920.5), egg white GGLEPINFQTAADQAR (844.4 > 666.3), peanut TANELNLLILR (635.4 > 741.5), and soybean EAFGVNMQIVR.*

The strategy was evaluated in terms of the linearity of the calibration curve (R^2) and the RSD between samples spiked (n=8, 1 replicate per matrix) at the highest concentration (20 x LOQ), with IS 1 and IS 2 internal standard correction in order to test the possibility of using a single calibration curve for the quantification of allergens in foodstuffs.

The regression coefficient (R^2) was higher than 0.99 for 27 linear regressions out of 31 when no internal standard was used, but the use of internal standard correction with IS 1 or IS 2 raised this number to 29 or 28, respectively.

As shown in Figure 2, the calibration curves obtained after correction with either IS 1 or IS 2 did not coincide for the eight matrices tested. Consequently, it proved impossible to use a single calibration curve for all the targeted food matrices in the case of milk, egg, soy, and peanut peptides. Considering the eight matrices, the relative standard deviation (RSD) calculated at 20 x LOQ ranged from 27% to 43% and from 112% to 74% for the 4 target allergens after IS 1 and IS 2 correction, respectively. The RSD was lower after IS 1 correction than after IS 2 correction (**Supplementary material – Table 2**).

As shown in Figure 2, after correction of peptide peak areas with IS 1, the calibration curves for mayonnaise and sauce appeared separate from the other calibration curves. We thus propose distinguishing two groups of matrices: those with a carbohydrate content higher than 50% (jam, compote, cookie, chocolate, and spices) and those with a fat content higher than 50% (mayonnaise and sauce). When only the group of matrices containing more than 50% carbohydrate was considered, the RSD dropped to the 6% to26 % range (calculated on the basis of the highest level of concentration after IS 1 correction) instead of the initial 27% to 43% for the eight matrices (Supplementary material – Table 2). As the RSD was higher than 20% (SMPR 2016.002), a single calibration curve per group of matrices cannot be used for the quantification of allergens in food (Paez et al., 2016).

.*3.4.2 Standard addition with labeled internal standard correction*

The standard addition strategy consists in adding known amounts of standard protein working solution to a food sample before extraction, in order to determine the initial allergen concentrations. The eight matrices were spiked at the LOQ (C1, n=6) and at 10 x LOQ (C2, n=6): per kg of food product, 0.5 mg and 5 mg for milk proteins, 0.75 mg and 7.5 mg for egg proteins, 5 mg and 50 mg for soy proteins, and 2.5 mg and 25 mg for peanut proteins. On these spiked samples, standard addition was performed at LOQ, 2 x LOQ, 5 x LOQ, 10 x LOQ, and 20 x LOQ, corresponding to 0, 0.5, 1, 2.5, 5,

and 10 mg/kg for milk proteins, 0, 0.75, 1.5, 3.75, 7.5, and 15 mg/kg for egg proteins, 0, 5, 10, 25, 50, and 100 mg/kg for soy proteins and 0, 2.5, 5, 12.5, 25, and 50 mg/kg for peanut proteins (**Figure 3**).

Proteins were detected and quantified in the matrices spiked at both levels, using two strategies: standard addition without and with correction of peptide peak areas with an internal standard (IS 1 or IS 2).

The RSD of the recovery (without or with labeled peptide correction) was calculated on three independent replicates of compote spiked at 10 x LOQ (C2).

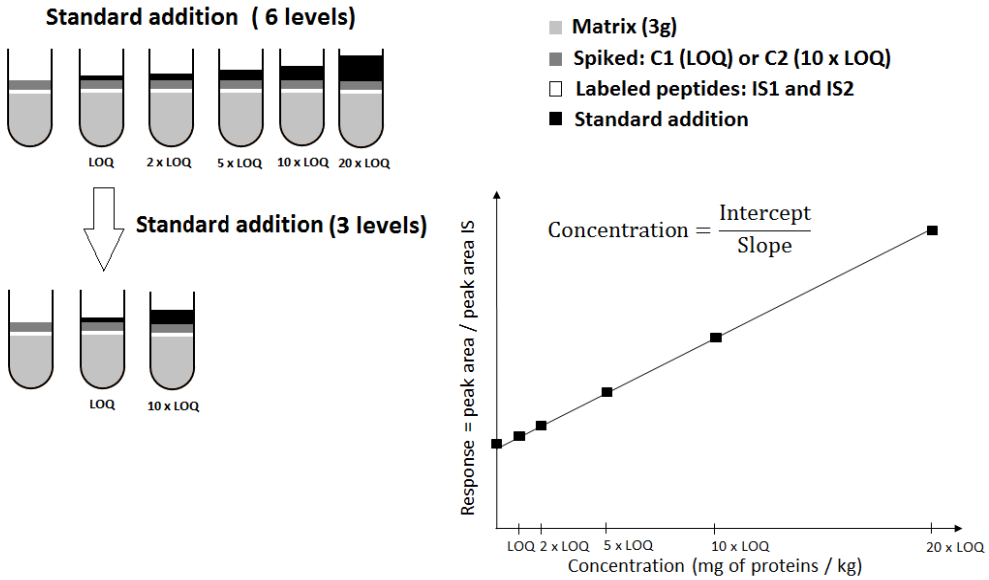


Figure 3: *Strategy for quantifying allergens in foodstuffs: standard addition + labeled internal standard IS 1 or IS 2. Quantification at six levels of concentration was compared to quantification at 3 levels (sample, sample spiked at the LOQ, and sample spiked at 10 x LOQ), the goal being to reduce the number of samples for the development of a routine method.*

- **Standard addition : 6 concentration levels**

The slope and intercept of the regression line obtained without internal standard correction and after correction with IS 1 and IS 2 were used to determine the initial concentrations of milk, egg, soy, and peanut allergens. As shown in Figure 3, the concentration was calculated by dividing the intercept by the slope (**Supplementary material – Tables 3 A and B**). Recoveries were calculated by dividing the estimated concentration by the theoretical concentration and multiplying by 100. Without correction with an internal standard, 35.0% of the calculated recoveries (21.7% at C1 and 13.3% at C2) were outside the range [60 – 120%] specified by the AOAC guideline SMPR 2016.002 (**Figure 4**). Correction with IS 1 and IS 2 allowed significantly reducing the percentage of recoveries failing to comply with the AOAC specification, to 18.3% (15.0% C1 and 3.3 % C2) and 16.7% (10.0% C1 and 6.7% C2), respectively.

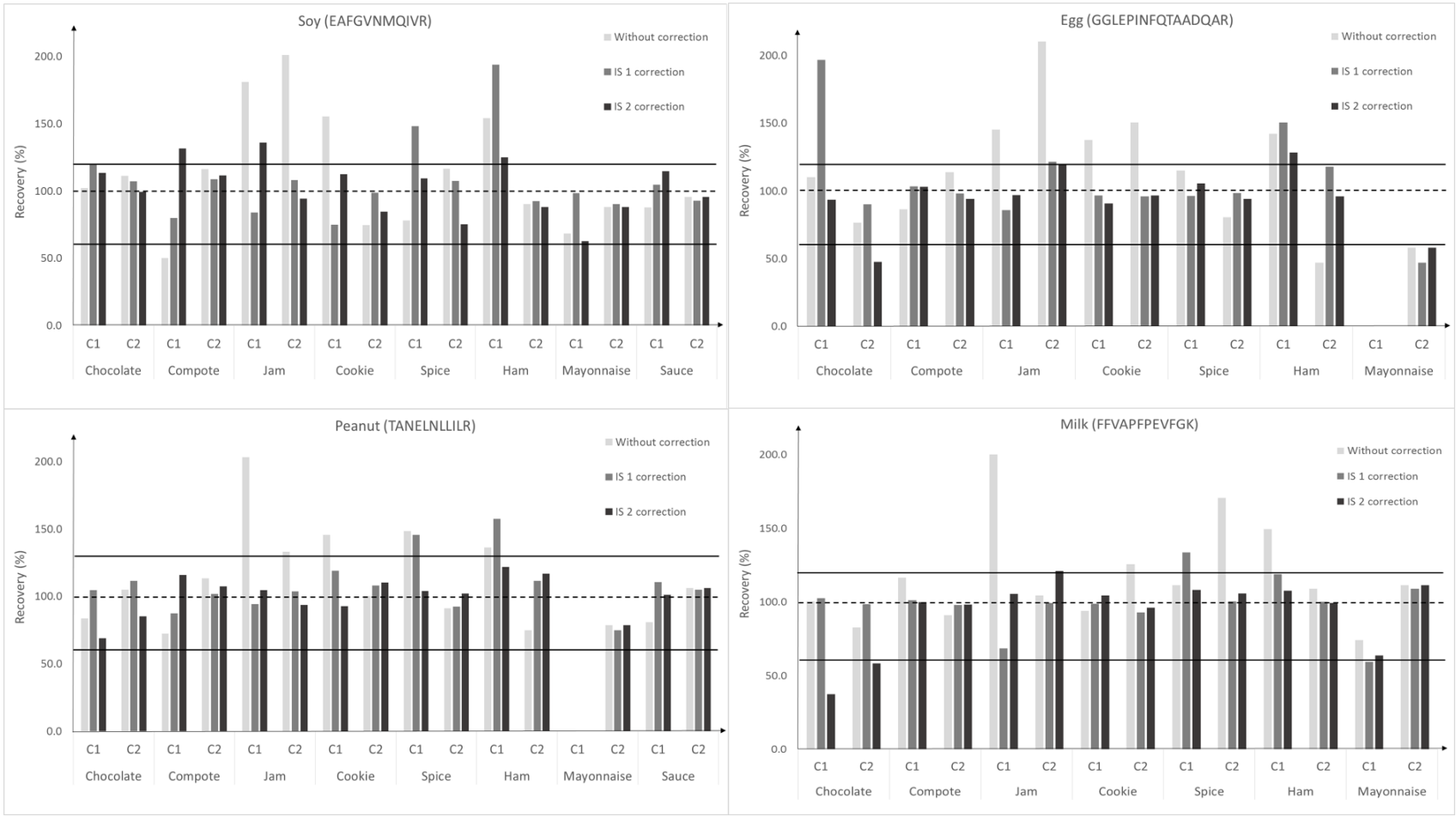


Figure 4: *Recoveries obtained by standard addition (6 levels of concentration) for soy, peanut, egg, and milk peptides added at the LOQ (C1) and at 10 x LOQ (C2) without internal standard correction, with labeled IS 1 peptide correction (FFVAPFPEVFGK[$^{13}C_6{}^{15}N_2$] (milk), GGLEPINF[D_5]QTAADQAR (egg), TANELNLLIL[$^{13}C_6{}^{15}N$]R (peanut), EAFGV[D_8]NMQIVR (soy)); and with long peptide IS 2 correction (GRFF**V**[$^{13}C_5{}^{15}N$]APFPEVFGKGG**L**[$^{13}C_6{}^{15}N$] EPINFQTAADQARGS (milk and egg) and GREAFG**V**[$^{13}C_5{}^{15}N$]NMQIVRTANE**L**[$^{13}C_6{}^{15}N$]NLLILRGS (soy and peanut). The recovery range specified by AOAC [60-120%] is delimited by two black lines.*

- **Calculation of the RSD between recoveries (n=3)**

The recoveries obtained for the three technical replicates of compote matrix spiked at C2 with IS 1 or IS 2 correction and without correction were compared (Supplementary material – Tables 4 A and B). Without internal standard correction, recoveries (n=3) were sometimes outside the range specified by the AOAC: milk (116 ± 14%), egg (126 ± 24%), soy (129 ± 25%), and peanut (115 ± 18%). Correction of peptide peak areas with IS 1 or IS 2 labeled peptides allowed respecting the AOAC specification, decreasing the relative standard deviation between replicates and giving rise to recoveries 103 ± 5% for milk, 103 ± 4% for egg, 107 ± 4% soy, and (100 ± 1%) for peanut with IS 1 correction and 103 ± 4% for milk, 105 ± 8% for egg, 111 ± 1% for soy, and 107 ± 1% for peanut with correction for IS 2.

The use of either type of labeled peptides made it possible to decrease significantly the percentage of out-of-range recoveries. At the LOQ, however, this percentage remained high. Some analytical regulations such as SANTE/11813/2017, concerning pesticide residue analysis in food and feed, recommend tolerating a [50 – 120%] recovery range at the LOQ.

- **Standard addition: 3 levels of concentration**

For a routine laboratory, standard addition should ideally be done with a limited number of spiking levels. Therefore, recoveries were also calculated for only three calibration points (0, 0.5 and 5 mg/kg for milk proteins, 0, 0.75 and 7.5 mg/kg for egg proteins, 0, 5 and 50 mg/kg for soy proteins, and 0, 2.5 and 25 mg/kg for peanut proteins). Without internal standard correction, 50.0% of the recoveries (27.4% at C1 and 22.6% at C2) were outside the range [60 – 120%] specified in AOAC guideline SMPR 2016.002 (Figure 4). Correcting with IS 1 or IS 2 made it possible to reduce the percentage of out-of-range to 13.3% (11.7% at C1 and 1.7% at C2) or 18.3% (13.3% at C1 and 5.0% at C2), respectively (Supplementary material – Tables 5 A and B). After correction of peak areas with the help of labeled peptides, the percentage of out-of-AOAC-range recoveries was similar to that obtained with 6 calibration points. In order to reduce the time of analysis (3 samples instead of 6), standard addition to matrices spiked with a standard at the 1 x and 10 x the LOQ was retained for the quantification of real samples.

3.5 Quantification of peanut in chocolate dessert samples

The standard addition strategy combined with the inclusion of IS 1 or IS 2 labeled peptides was used to quantify allergens in incurred chocolate desserts. Chocolate desserts containing 0, 2, 4, 10, and 30 mg peanut proteins per kg were spiked with peanut proteins at 0, 2.5, and 25 mg/kg. The corresponding MRM chromatograms are presented in Figure 5.

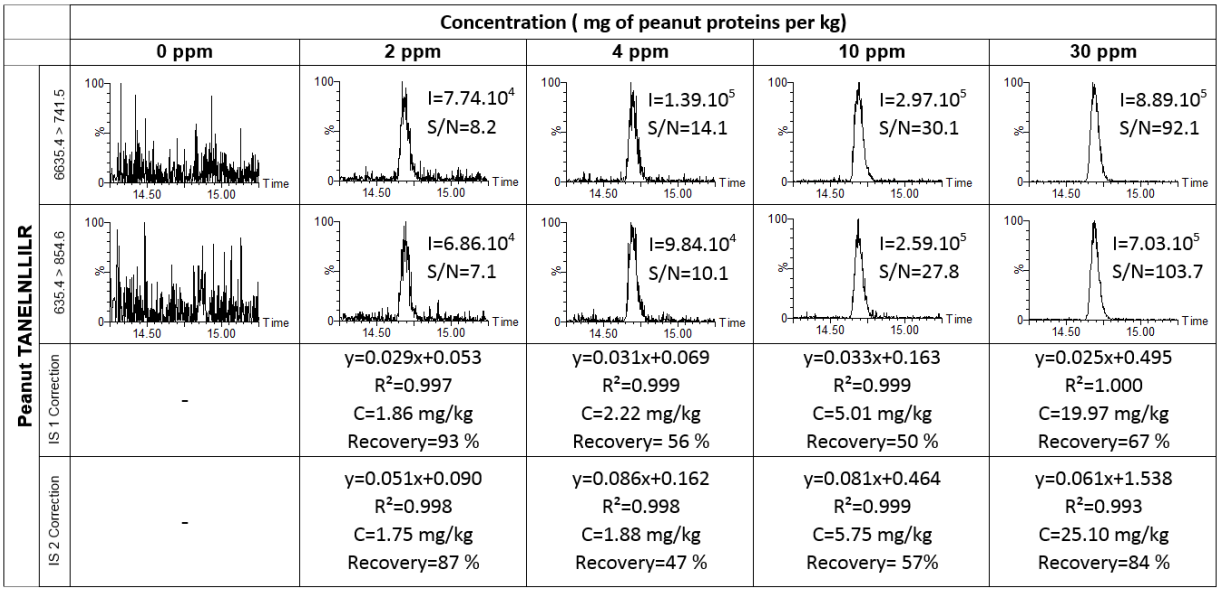


Figure 5: *Detection and quantification of peanut with TANELNLLILR peptide in iFAAM chocolate dessert matrices containing 0, 2, 4, 10, and 30 mg peanut proteins per kg. Recovery was calculated with IS 1 and IS 2 labeled peptide correction.*

The developed method can detect peanut in chocolate dessert at 2 mg peanut proteins per kg with a S/N ratio higher than 3. The slope and the intercept of calibration curves obtained for peanut peptide (TANELNLLILR) in chocolate desserts were used to determine the recovery. The recovery from incurred chocolate desserts ranged from 50 to 93% with IS 1 correction and from 47 to 87% with IS 2 correction. With IS 1 and IS 2 correction, respectively the recoveries previously determined for spiked chocolate were 104.5% and 69% at the LOQ and 111.6% and 85.1% at 10 x LOQ. As shown previously, the recovery range [60 – 120%] set by a panel of experts in the AOAC guideline can be hard to reach for incurred food products (Newsome and Scholl 2013; Sayers et al. 2018), but a calibration curve prepared under conditions similar to those used for samples, as in Gu et al., gives better recoveries (60.1 to 92.4% for milk, soy, peanut and tree nut proteins). This is totally unrealistic for routine laboratories, however, because of the quantity of samples to be analyzes (Gu et al. 2018). Furthermore, in routine laboratories the process conditions and in some instances the recipe are not specified on the samples received, so the product cannot be reproduced.

4 Conclusion

The developed method can detect 10 allergens (egg, milk, soy, peanut, almond, hazelnut, walnut, pecan nuts, cashew, and pistachio) in eight matrices belonging to different food product categories (high in fat, carbohydrate, protein, tannins, or polyphenols). Developed for a routine laboratory, the method uses a single protocol to detect 10 allergens within a day. We have previously reported sensitive methods for processed samples (sauce heated at 95°C for 45 min and cookie baked at 180°C for 18 min) and incurred samples (chocolate and banana ice cream) (Planque et al. 2017; Planque et

al. 2016), but in high-fat matrices or spices, some allergens were not detected at the determined LOQs after standard addition to matrices spiked with the target allergens at the LOQ. The complexity of some food products leads to interferences influencing the sensitivity of MRM signals. We have evaluated several factors liable to influence the rate of false positives or negatives, such as the retention time, the S/N ratio, and the relative ion intensity. On the basis of the results obtained, we recommend using the following criteria for allergen detection: signal-to-noise ratios above 10 and 3 for the first and second transitions, respectively, a 2.5% retention time deviation between matrix-matched samples and a relative ion intensity deviation according to guideline SANTE/11813/2017. Furthermore, the selection of marker peptides and the determination of method sensitivity (LOD, LOQ) should always be done with processed and incurred food products because of the impact of the thermal process on allergen detection. Standard addition by spiking the matrices with target allergens at the LOQ is imperative to ensure detection of allergens at the LOQ and to decrease the rates of false positives and negatives.

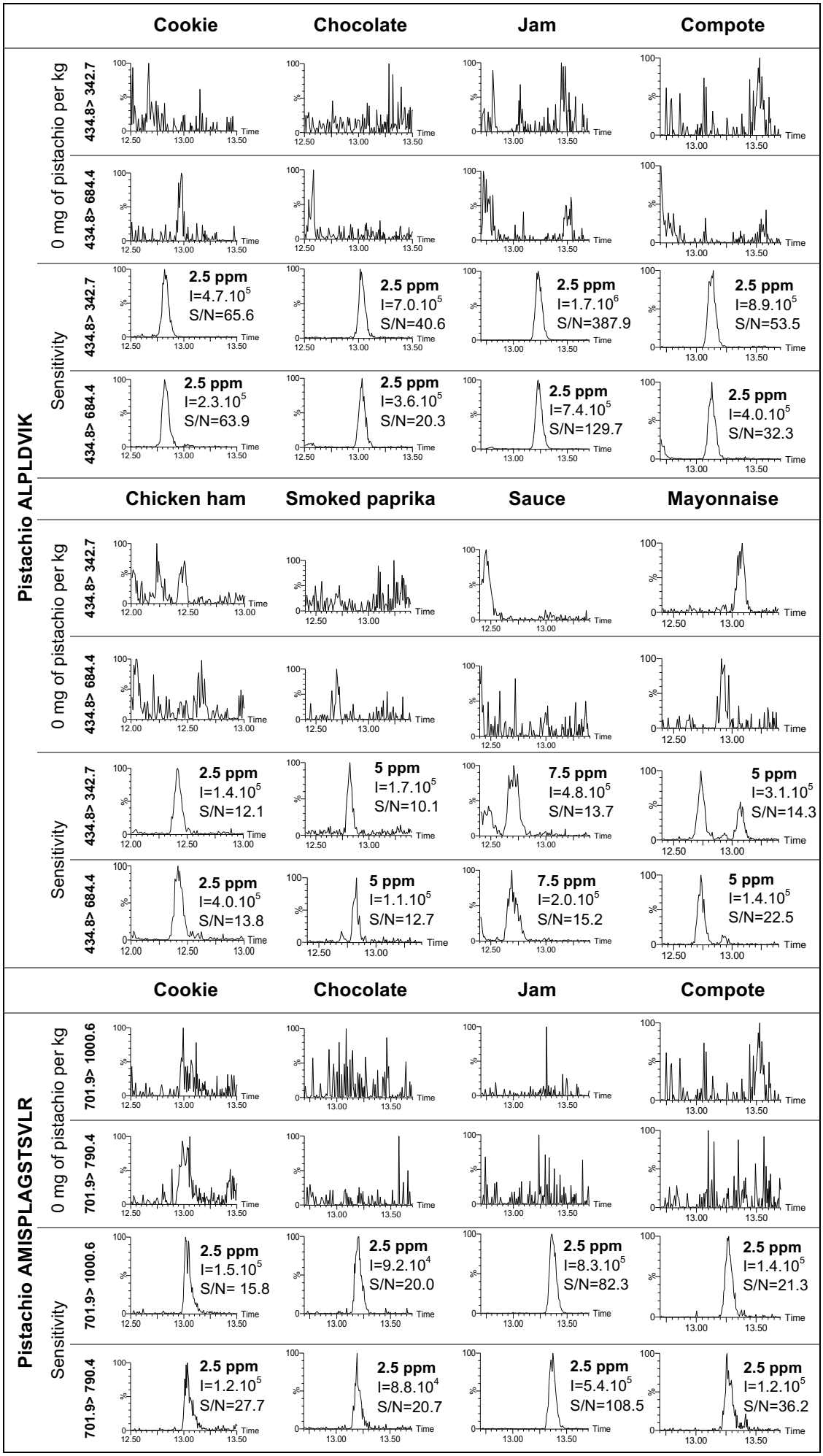
In the second part of this work, we have compared two strategies for quantifying milk, soy, peanut, and egg allergens in the eight food products. For a routine laboratory, the ideal quantification strategy involves the use of a single calibration curve. Yet neither the use of labeled peptides (IS 1) nor the use of long isotope-labeled peptides (IS 2) enabled us to use a single calibration curve for the quantification of all target allergens. The strategy combining the use of a labeled internal standard with standard addition appears promising, since recoveries of 81.7% (IS 1 correction) and 83.3% (IS 2 correction) were determined at two concentrations (LOQ and 10 x LOQ) in eight matrices for milk, egg, soy, and peanut allergens and since these values fall within the range [60 -120%] specified in SMPR 2016.002. We have also used the same strategy to quantify peanut proteins in an incurred chocolate matrix containing 0, 2, 4, 10, and 30 mg peanut proteins per kg, and recoveries obtained with IS 1 correction were between 50% and 93% and those obtained with IS 2 correction were between 47 and 87%. Unexpectedly, we observed no improvement of recovery with long isotope-labeled peptides combining milk with egg or peanut with soy peptides. On the basis of the results obtained, we recommend tolerating a wider recovery range at the LOQ. To reach the goal of using a single calibration curve, the use of labeled proteins should next be tested, but our strategy combining standard addition with labeled peptides is already a very efficient alternative allowing allergen quantification in all kinds of foodstuffs with good recovery.

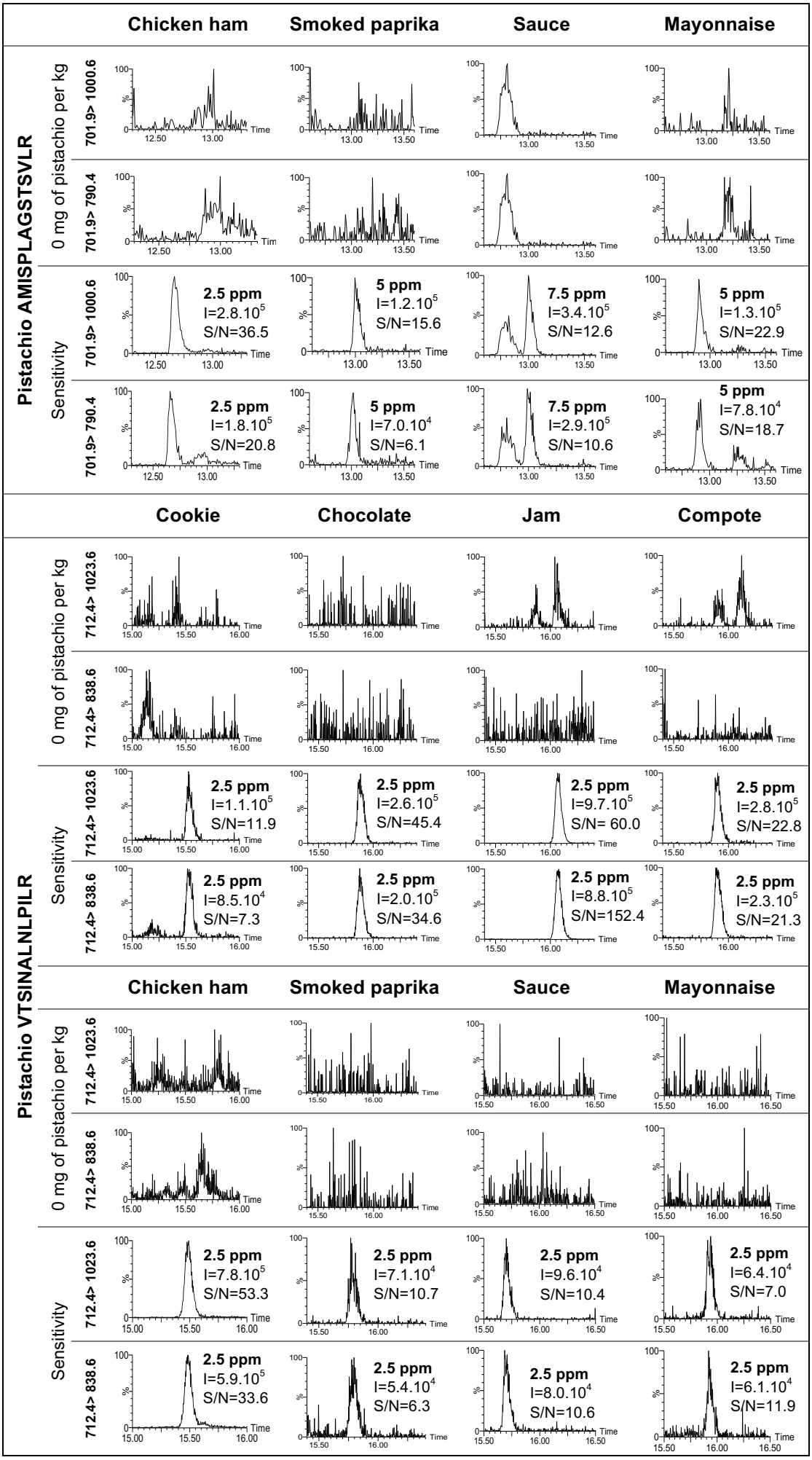
Acknowledgments

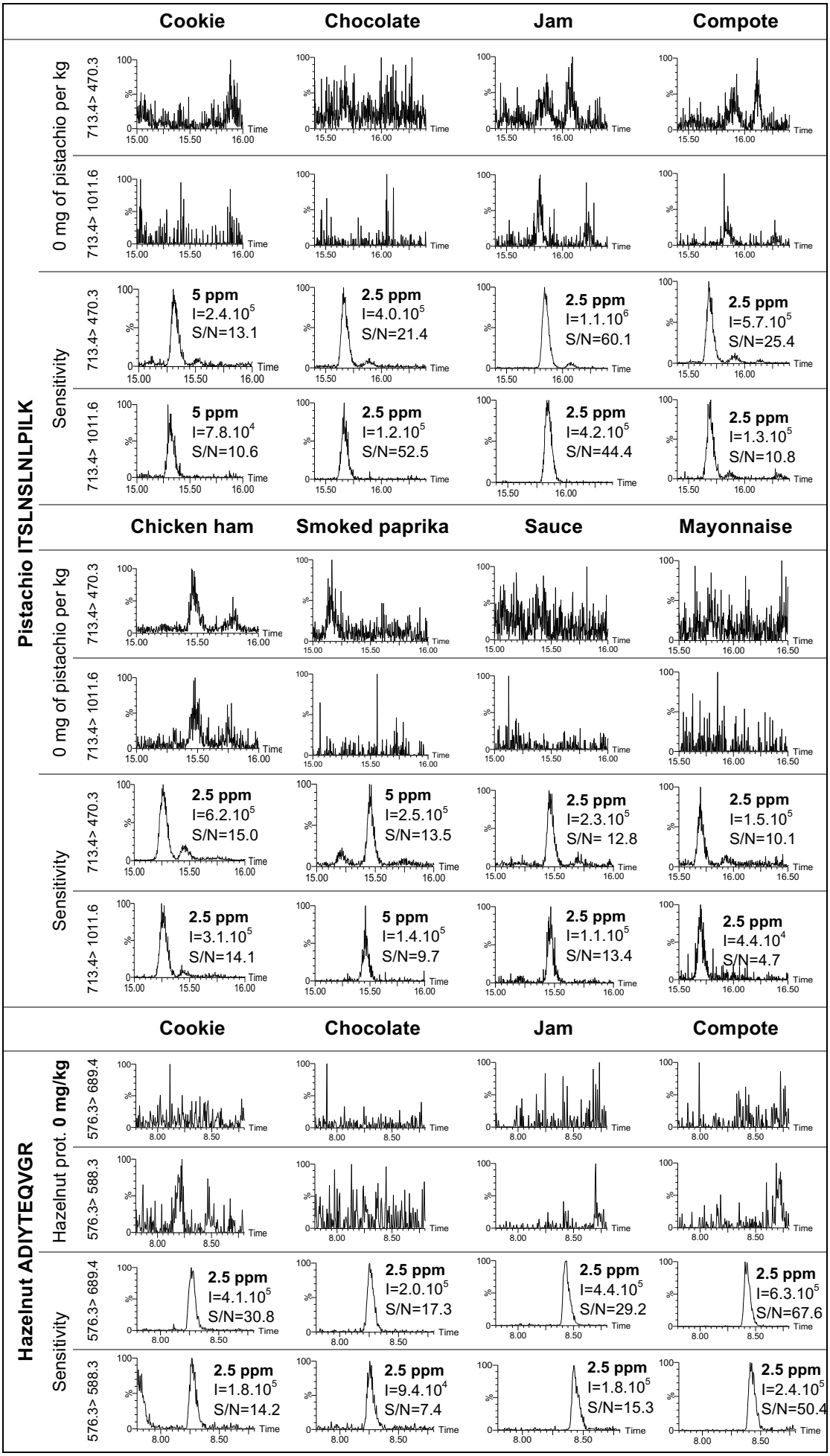
We thank Prof. Clare Mills and Prof. Victoria Lee for permission to use the chocolate dessert matrices produced by the University of Manchester in the framework of the “Integrated approaches to Food Allergen and Allergy Management” project (iFAAM). We thank the Waters Corporation, the Walloon Region (FirstDoCA project: Allermass convention 1217881), and the Technological Platform Mass

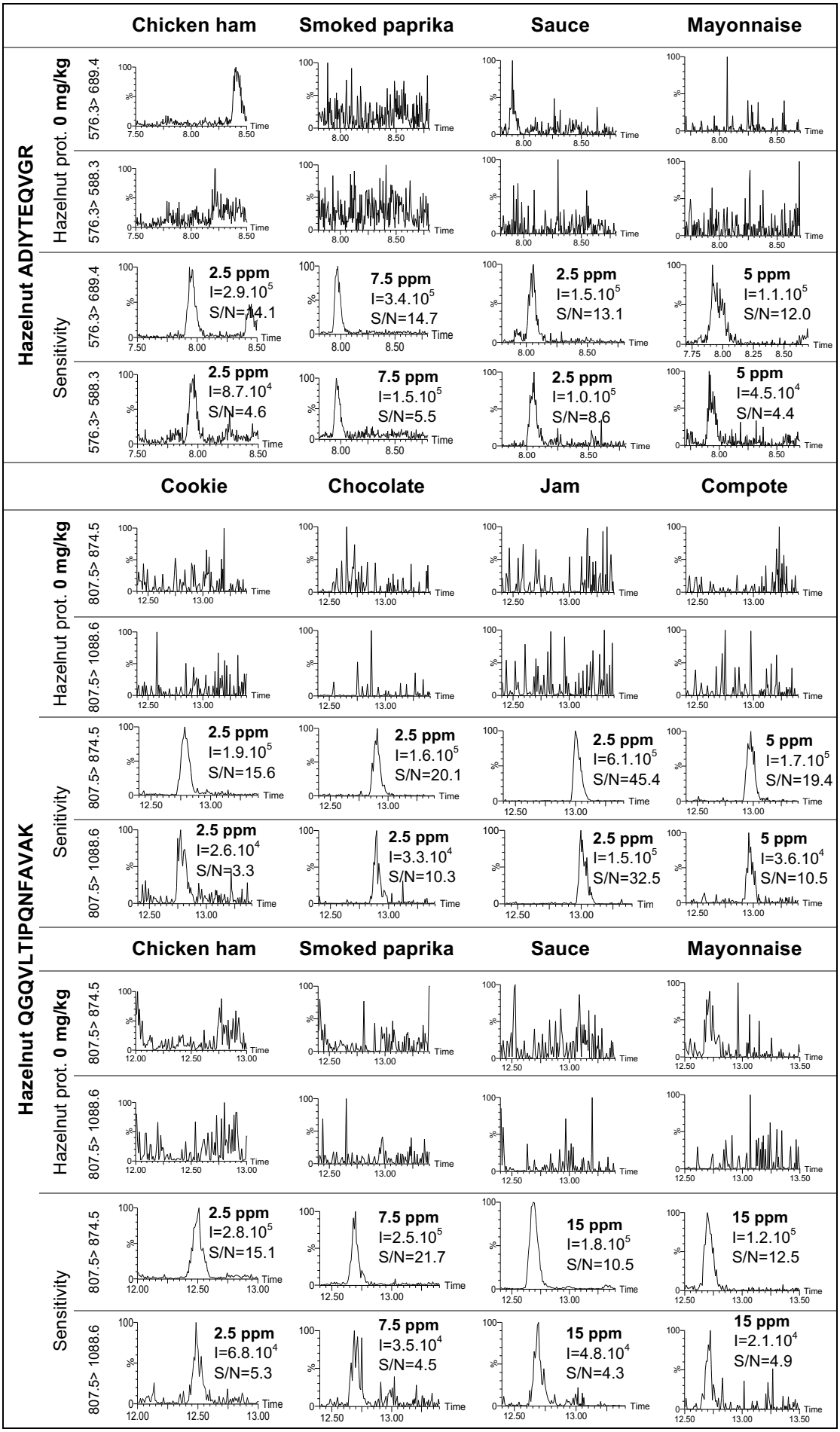
Spectrometry Service of the University of Namur, Belgium (MaSUN, UNamur) for scientific support and for their financial contributions to this project.

Supplementary material Figure 1: *Chromatograms of the two highest multiple reaction monitoring MRM transitions of milk, egg, soy, peanut, pistachio, hazelnut, cashew, almond, walnut, and pecan nut allergens. Data for non-contaminated matrices (0 mg/kg) and for matrices spiked at the limit of quantification are presented without smoothing.*


Pistachio ALPLDVIK
Cookie
Chocolate
Jam
Compote
0 mg of pistachio per kg
Sensitivity
434.8> 342.7
434.8> 684.4
2.5 ppm I=4.7.10^5 S/N=65.6
2.5 ppm I=7.0.10^5 S/N=40.6
2.5 ppm I=1.7.10^6 S/N=387.9
2.5 ppm I=8.9.10^5 S/N=53.5
2.5 ppm I=2.3.10^5 S/N=63.9
2.5 ppm I=3.6.10^5 S/N=20.3
2.5 ppm I=7.4.10^5 S/N=129.7
2.5 ppm I=4.0.10^5 S/N=32.3
Chicken ham
Smoked paprika
Sauce
Mayonnaise
2.5 ppm I=1.4.10^5 S/N=12.1
5 ppm I=1.7.10^5 S/N=10.1
7.5 ppm I=4.8.10^5 S/N=13.7
5 ppm I=3.1.10^5 S/N=14.3
2.5 ppm I=4.0.10^5 S/N=13.8
5 ppm I=1.1.10^5 S/N=12.7
7.5 ppm I=2.0.10^5 S/N=15.2
5 ppm I=1.4.10^5 S/N=22.5
Pistachio AMISPLAGSTSVLR
Cookie
Chocolate
Jam
Compote
0 mg of pistachio per kg
Sensitivity
701.9> 1000.6
701.9> 790.4
2.5 ppm I=1.5.10^5 S/N= 15.8
2.5 ppm I=9.2.10^4 S/N=20.0
2.5 ppm I=8.3.10^5 S/N=82.3
2.5 ppm I=1.4.10^5 S/N=21.3
2.5 ppm I=1.2.10^5 S/N=27.7
2.5 ppm I=8.8.10^4 S/N=20.7
2.5 ppm I=5.4.10^5 S/N=108.5
2.5 ppm I=1.2.10^5 S/N=36.2


Chicken ham
Smoked paprika
Sauce
Mayonnaise
Pistachio AMISPLAGSTSVLR
0 mg of pistachio per kg
701.9> 1000.6
701.9> 790.4
Sensitivity
2.5 ppm I=2.8.10^5 S/N=36.5
5 ppm I=1.2.10^5 S/N=15.6
7.5 ppm I=3.4.10^5 S/N=12.6
5 ppm I=1.3.10^5 S/N=22.9
2.5 ppm I=1.8.10^5 S/N=20.8
5 ppm I=7.0.10^4 S/N=6.1
7.5 ppm I=2.9.10^5 S/N=10.6
5 ppm I=7.8.10^4 S/N=18.7
Cookie
Chocolate
Jam
Compote
Pistachio VTSINALNLPILR
712.4> 1023.6
712.4> 838.6
2.5 ppm I=1.1.10^5 S/N=11.9
2.5 ppm I=2.6.10^5 S/N=45.4
2.5 ppm I=9.7.10^5 S/N= 60.0
2.5 ppm I=2.8.10^5 S/N=22.8
2.5 ppm I=8.5.10^4 S/N=7.3
2.5 ppm I=2.0.10^5 S/N=34.6
2.5 ppm I=8.8.10^5 S/N=152.4
2.5 ppm I=2.3.10^5 S/N=21.3
Chicken ham
Smoked paprika
Sauce
Mayonnaise
2.5 ppm I=7.8.10^5 S/N=53.3
2.5 ppm I=7.1.10^4 S/N=10.7
2.5 ppm I=9.6.10^4 S/N=10.4
2.5 ppm I=6.4.10^4 S/N=7.0
2.5 ppm I=5.9.10^5 S/N=33.6
2.5 ppm I=5.4.10^4 S/N=6.3
2.5 ppm I=8.0.10^4 S/N=10.6
2.5 ppm I=6.1.10^4 S/N=11.9


Cookie
Chocolate
Jam
Compote
Pistachio ITSLNSLNLPILK
0 mg of pistachio per kg
713.4> 470.3
713.4> 1011.6
Sensitivity
5 ppm I=2.4.10^5 S/N=13.1
2.5 ppm I=4.0.10^5 S/N=21.4
2.5 ppm I=1.1.10^6 S/N=60.1
2.5 ppm I=5.7.10^5 S/N=25.4
5 ppm I=7.8.10^4 S/N=10.6
2.5 ppm I=1.2.10^5 S/N=52.5
2.5 ppm I=4.2.10^5 S/N=44.4
2.5 ppm I=1.3.10^5 S/N=10.8
Chicken ham
Smoked paprika
Sauce
Mayonnaise
2.5 ppm I=6.2.10^5 S/N=15.0
5 ppm I=2.5.10^5 S/N=13.5
2.5 ppm I=2.3.10^5 S/N= 12.8
2.5 ppm I=1.5.10^5 S/N=10.1
2.5 ppm I=3.1.10^5 S/N=14.1
5 ppm I=1.4.10^5 S/N=9.7
2.5 ppm I=1.1.10^5 S/N=13.4
2.5 ppm I=4.4.10^4 S/N=4.7
Hazelnut ADIYTEQVGR
Hazelnut prot. 0 mg/kg
576.3> 689.4
576.3> 588.3
2.5 ppm I=4.1.10^5 S/N=30.8
2.5 ppm I=2.0.10^5 S/N=17.3
2.5 ppm I=4.4.10^5 S/N=29.2
2.5 ppm I=6.3.10^5 S/N=67.6
2.5 ppm I=1.8.10^5 S/N=14.2
2.5 ppm I=9.4.10^4 S/N=7.4
2.5 ppm I=1.8.10^5 S/N=15.3
2.5 ppm I=2.4.10^5 S/N=50.4


Chicken ham
Smoked paprika
Sauce
Mayonnaise
Hazelnut ADIYTEQVGR
Hazelnut prot. 0 mg/kg
576.3> 689.4
576.3> 588.3
Sensitivity
2.5 ppm I=2.9.10^5 S/N=14.1
7.5 ppm I=3.4.10^5 S/N=14.7
2.5 ppm I=1.5.10^5 S/N=13.1
5 ppm I=1.1.10^5 S/N=12.0
2.5 ppm I=8.7.10^4 S/N=4.6
7.5 ppm I=1.5.10^5 S/N=5.5
2.5 ppm I=1.0.10^5 S/N=8.6
5 ppm I=4.5.10^4 S/N=4.4
Cookie
Chocolate
Jam
Compote
Hazelnut QGQVLTIPQNFAVAK
Hazelnut prot. 0 mg/kg
807.5> 874.5
807.5> 1088.6
Senitivity
2.5 ppm I=1.9.10^5 S/N=15.6
2.5 ppm I=1.6.10^5 S/N=20.1
2.5 ppm I=6.1.10^5 S/N=45.4
5 ppm I=1.7.10^5 S/N=19.4
2.5 ppm I=2.6.10^4 S/N=3.3
2.5 ppm I=3.3.10^4 S/N=10.3
2.5 ppm I=1.5.10^5 S/N=32.5
5 ppm I=3.6.10^4 S/N=10.5
Chicken ham
Smoked paprika
Sauce
Mayonnaise
Hazelnut prot. 0 mg/kg
807.5> 874.5
807.5> 1088.6
Sensitivity
2.5 ppm I=2.8.10^5 S/N=15.1
7.5 ppm I=2.5.10^5 S/N=21.7
15 ppm I=1.8.10^5 S/N=10.5
15 ppm I=1.2.10^5 S/N=12.5
2.5 ppm I=6.8.10^4 S/N=5.3
7.5 ppm I=3.5.10^4 S/N=4.5
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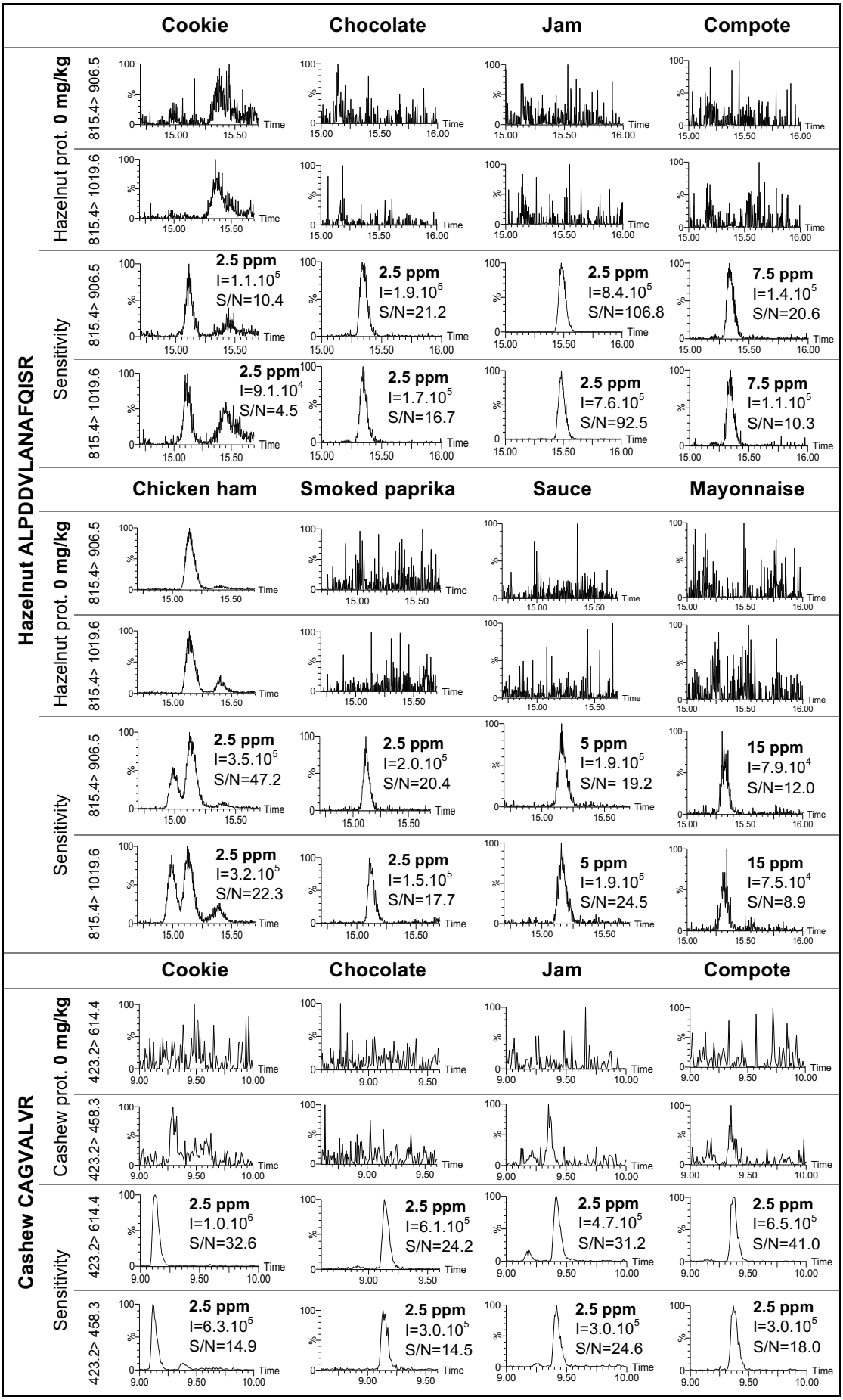
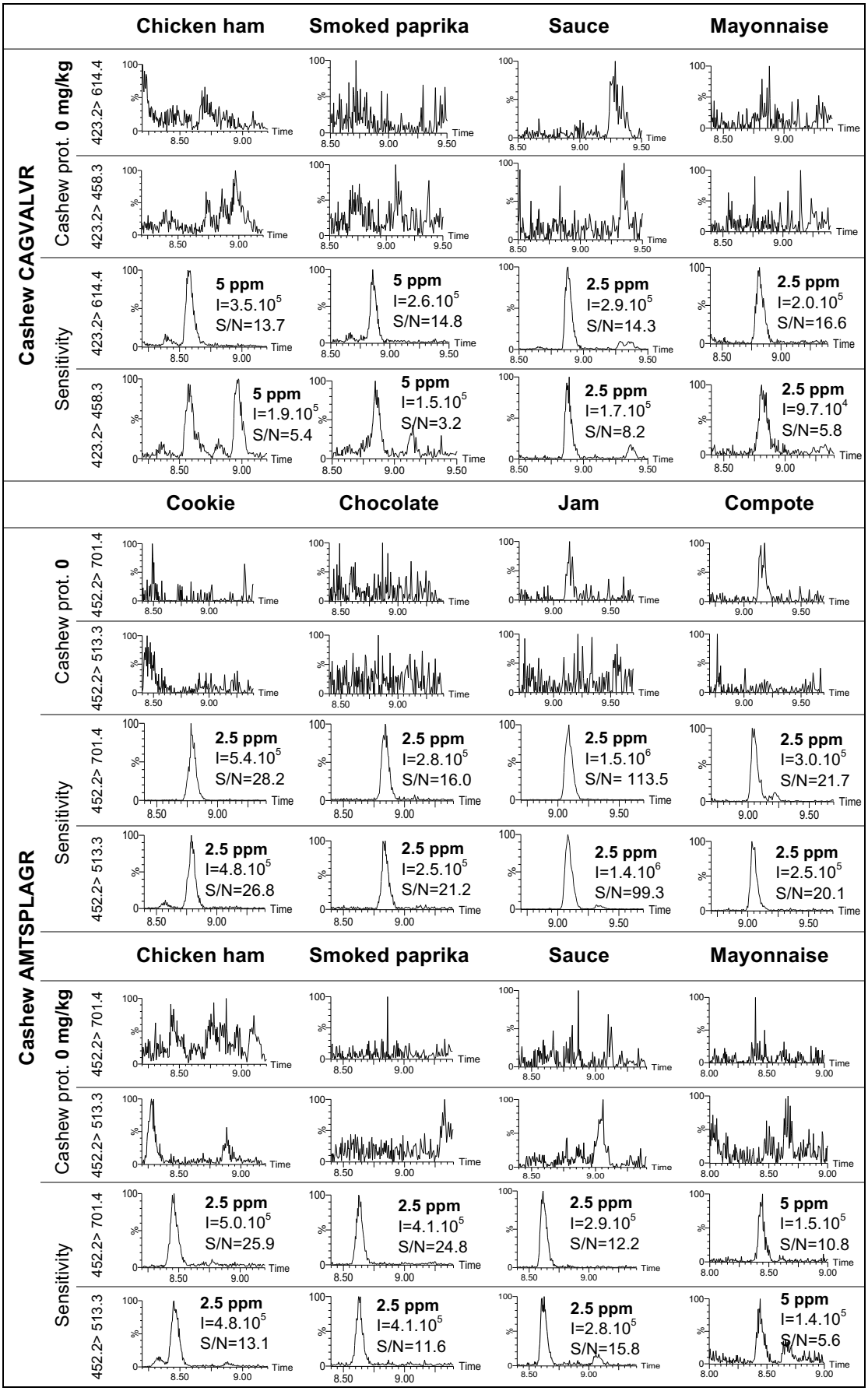
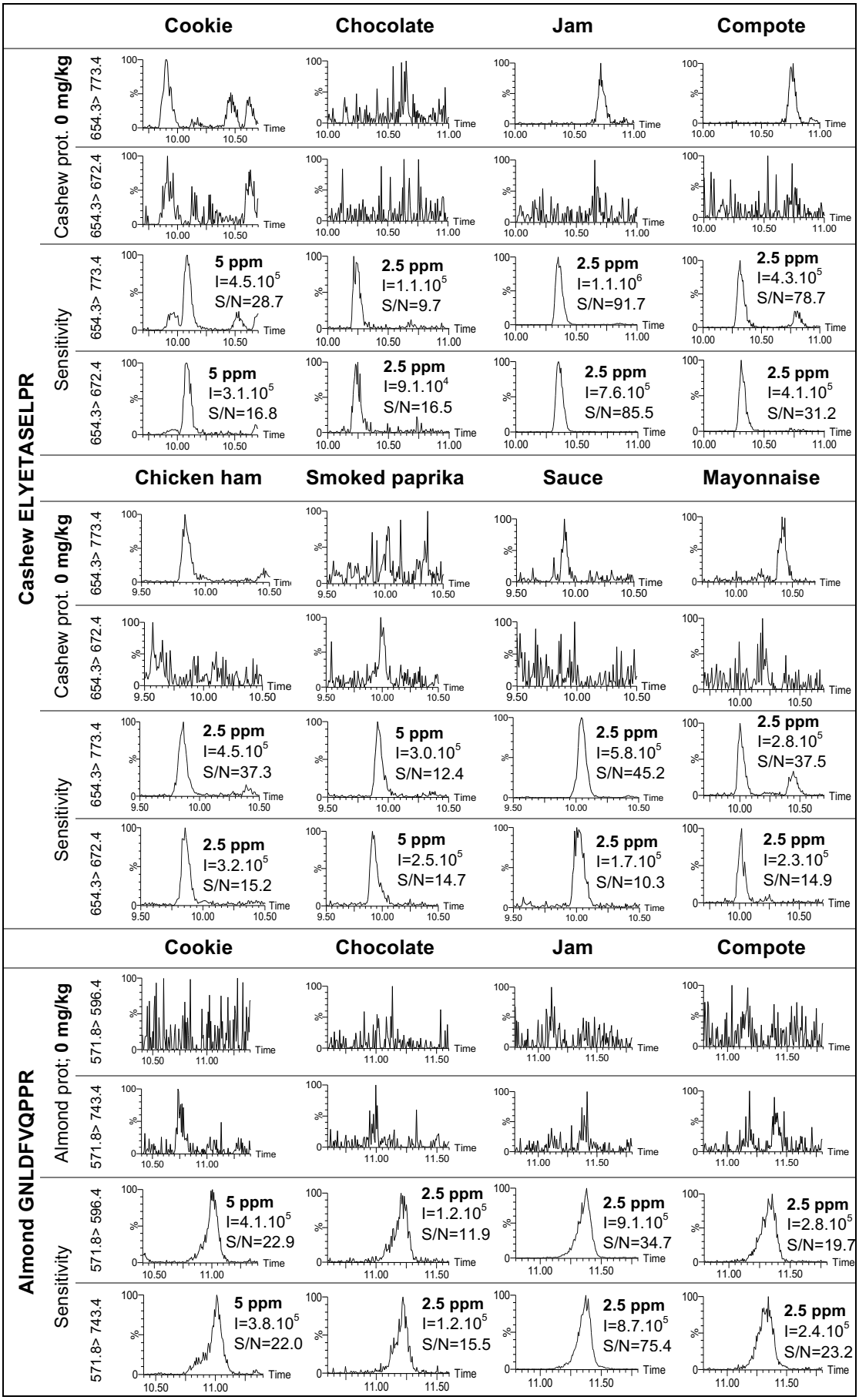
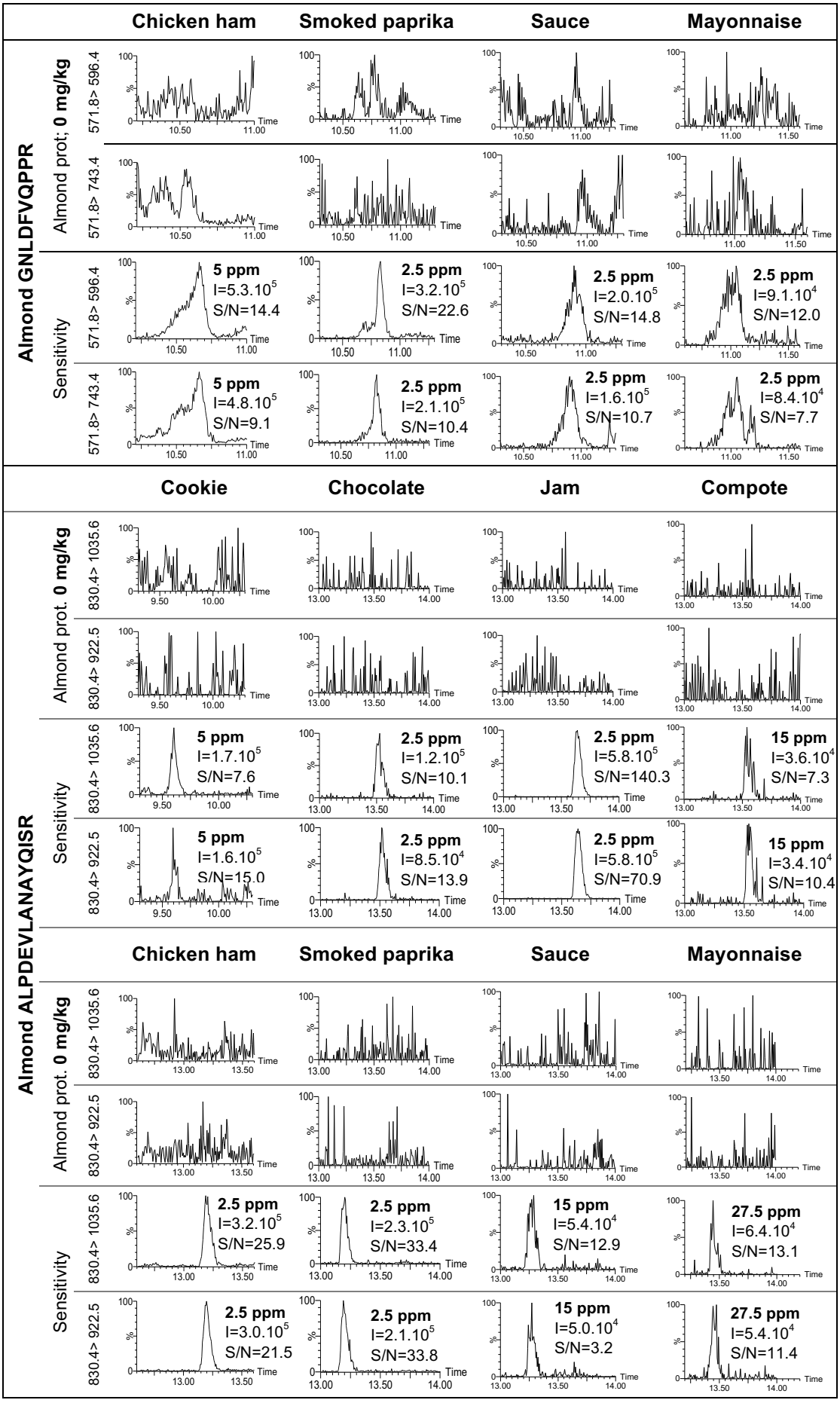
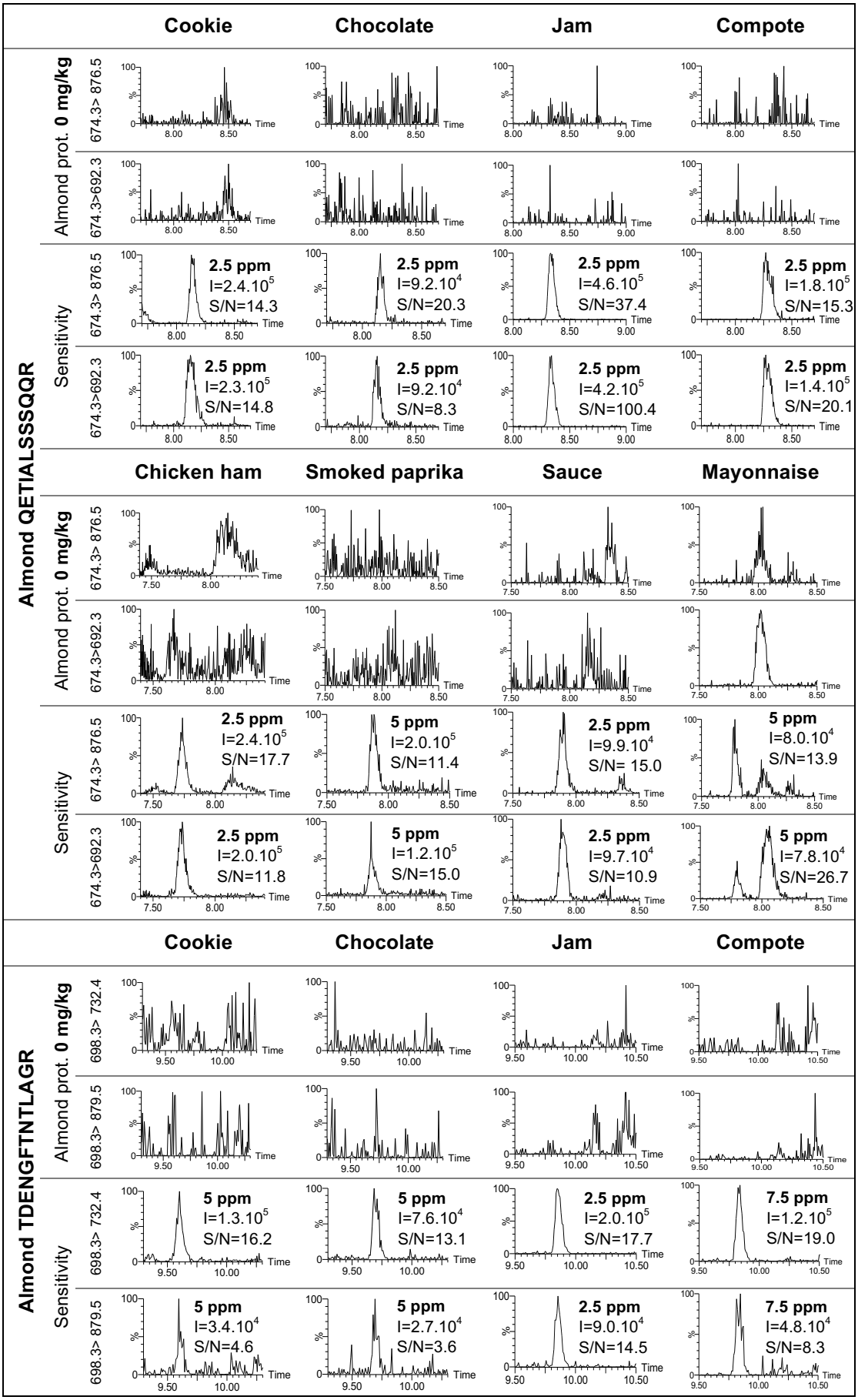
15 ppm I=2.1.10^4 S/N=4.9

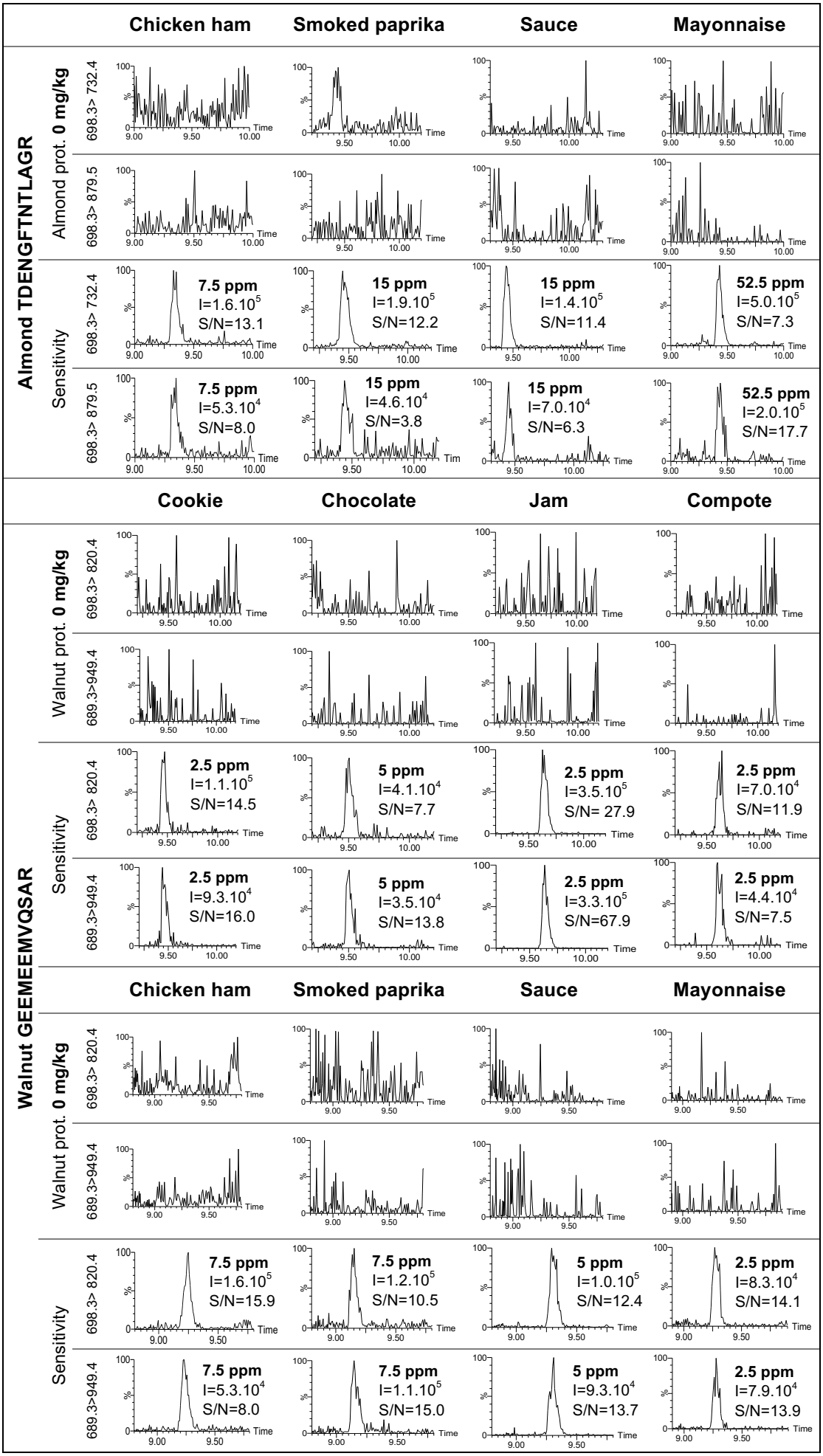

Cookie
Chocolate
Jam
Compote
Hazelnut ALPDDVLANAFQISR
Hazelnut prot. 0 mg/kg
815.4> 906.5
815.4> 1019.6
Sensitivity
2.5 ppm I=1.1.10^5 S/N=10.4
2.5 ppm I=1.9.10^5 S/N=21.2
2.5 ppm I=8.4.10^5 S/N=106.8
7.5 ppm I=1.4.10^5 S/N=20.6
2.5 ppm I=9.1.10^4 S/N=4.5
2.5 ppm I=1.7.10^5 S/N=16.7
2.5 ppm I=7.6.10^5 S/N=92.5
7.5 ppm I=1.1.10^5 S/N=10.3
Chicken ham
Smoked paprika
Sauce
Mayonnaise
2.5 ppm I=3.5.10^5 S/N=47.2
2.5 ppm I=2.0.10^5 S/N=20.4
5 ppm I=1.9.10^5 S/N= 19.2
15 ppm I=7.9.10^4 S/N=12.0
2.5 ppm I=3.2.10^5 S/N=22.3
2.5 ppm I=1.5.10^5 S/N=17.7
5 ppm I=1.9.10^5 S/N=24.5
15 ppm I=7.5.10^4 S/N=8.9
Cookie
Chocolate
Jam
Compote
Cashew CAGVALVR
Cashew prot. 0 mg/kg
423.2> 614.4
423.2> 458.3
Sensitivity
2.5 ppm I=1.0.10^6 S/N=32.6
2.5 ppm I=6.1.10^5 S/N=24.2
2.5 ppm I=4.7.10^5 S/N=31.2
2.5 ppm I=6.5.10^5 S/N=41.0
2.5 ppm I=6.3.10^5 S/N=14.9
2.5 ppm I=3.0.10^5 S/N=14.5
2.5 ppm I=3.0.10^5 S/N=24.6
2.5 ppm I=3.0.10^5 S/N=18.0

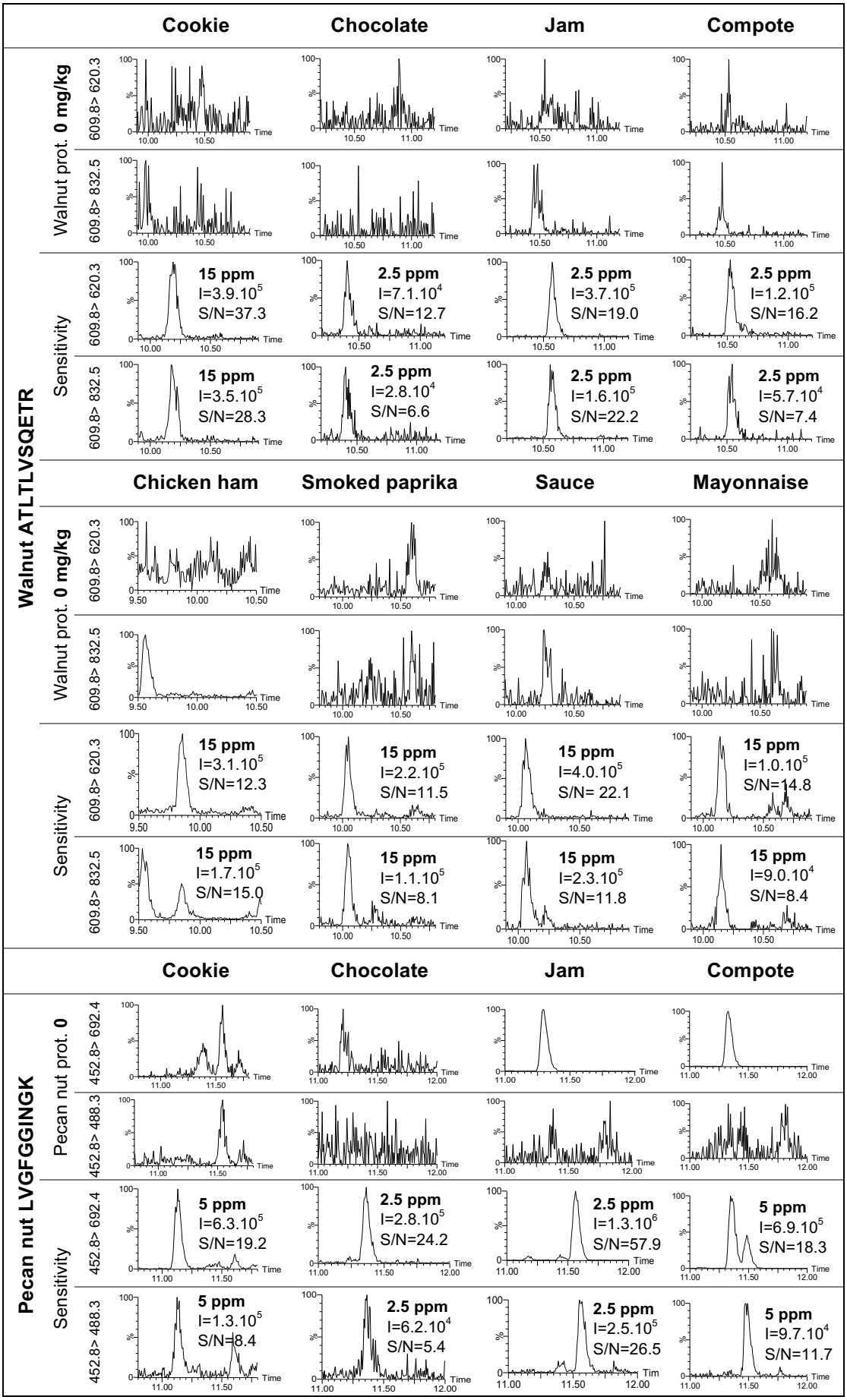

Cashew CAGVALVR
Chicken ham
Smoked paprika
Sauce
Mayonnaise
Cashew prot. 0 mg/kg
423.2> 614.4
423.2> 458.3
Sensitivity
5 ppm I=3.5.10^5 S/N=13.7
5 ppm I=2.6.10^5 S/N=14.8
2.5 ppm I=2.9.10^5 S/N=14.3
2.5 ppm I=2.0.10^5 S/N=16.6
5 ppm I=1.9.10^5 S/N=5.4
5 ppm I=1.5.10^5 S/N=3.2
2.5 ppm I=1.7.10^5 S/N=8.2
2.5 ppm I=9.7.10^4 S/N=5.8
Cashew AMTSPLAGR
Cookie
Chocolate
Jam
Compote
Cashew prot. 0
452.2> 701.4
452.2> 513.3
2.5 ppm I=5.4.10^5 S/N=28.2
2.5 ppm I=2.8.10^5 S/N=16.0
2.5 ppm I=1.5.10^6 S/N= 113.5
2.5 ppm I=3.0.10^5 S/N=21.7
2.5 ppm I=4.8.10^5 S/N=26.8
2.5 ppm I=2.5.10^5 S/N=21.2
2.5 ppm I=1.4.10^6 S/N=99.3
2.5 ppm I=2.5.10^5 S/N=20.1
Chicken ham
Smoked paprika
Sauce
Mayonnaise
Cashew prot. 0 mg/kg
452.2> 701.4
452.2> 513.3
Sensitivity
2.5 ppm I=5.0.10^5 S/N=25.9
2.5 ppm I=4.1.10^5 S/N=24.8
2.5 ppm I=2.9.10^5 S/N=12.2
5 ppm I=1.5.10^5 S/N=10.8
2.5 ppm I=4.8.10^5 S/N=13.1
2.5 ppm I=4.1.10^5 S/N=11.6
2.5 ppm I=2.8.10^5 S/N=15.8
5 ppm I=1.4.10^5 S/N=5.6
Time

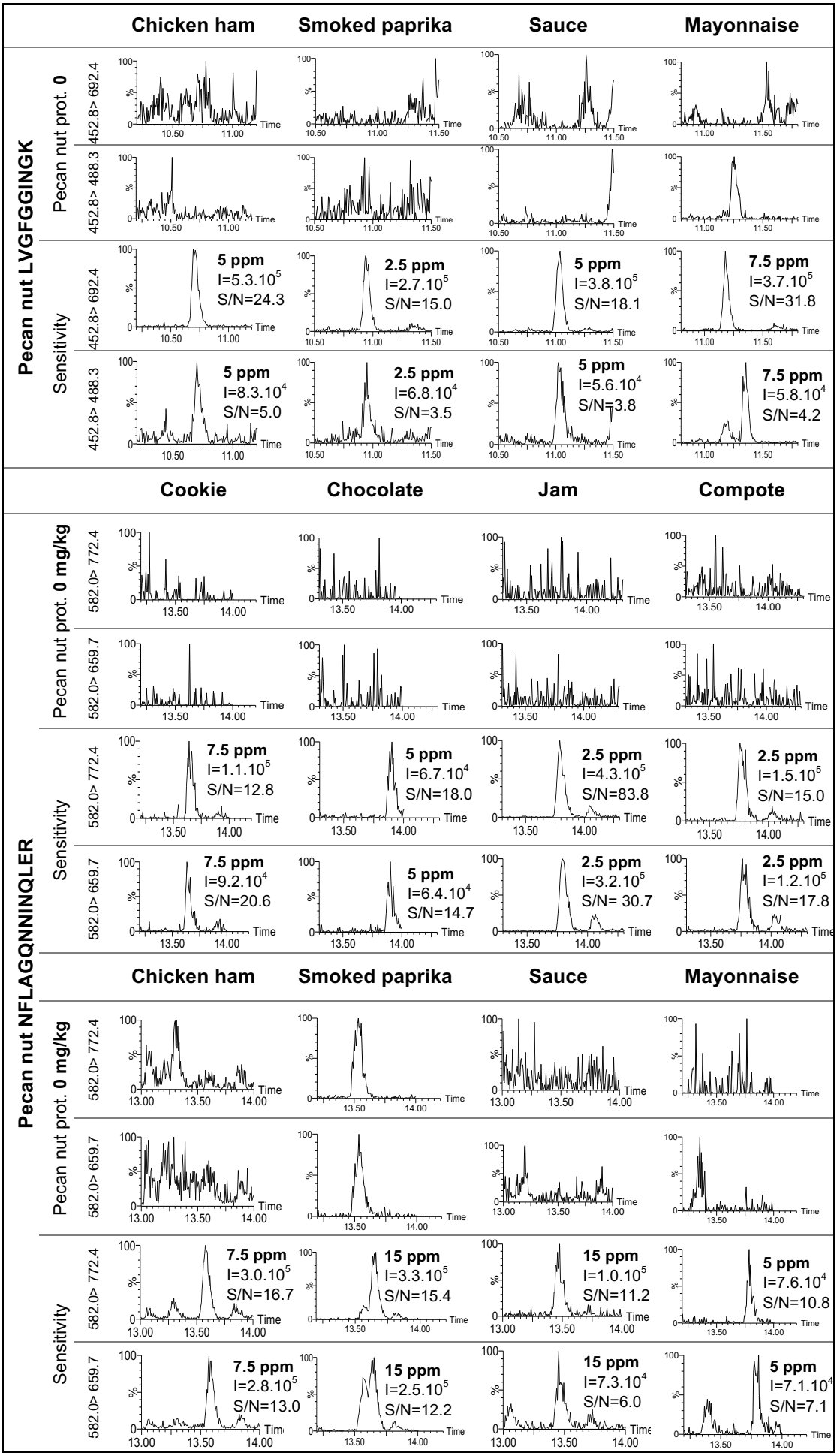

Cookie
Chocolate
Jam
Compote
Cashew ELYETASELPR
Cashew prot. 0 mg/kg
654.3> 773.4
654.3> 672.4
Sensitivity
5 ppm I=4.5.10^5 S/N=28.7
2.5 ppm I=1.1.10^5 S/N=9.7
2.5 ppm I=1.1.10^6 S/N=91.7
2.5 ppm I=4.3.10^5 S/N=78.7
5 ppm I=3.1.10^5 S/N=16.8
2.5 ppm I=9.1.10^4 S/N=16.5
2.5 ppm I=7.6.10^5 S/N=85.5
2.5 ppm I=4.1.10^5 S/N=31.2
Chicken ham
Smoked paprika
Sauce
Mayonnaise
2.5 ppm I=4.5.10^5 S/N=37.3
5 ppm I=3.0.10^5 S/N=12.4
2.5 ppm I=5.8.10^5 S/N=45.2
2.5 ppm I=2.8.10^5 S/N=37.5
2.5 ppm I=3.2.10^5 S/N=15.2
5 ppm I=2.5.10^5 S/N=14.7
2.5 ppm I=1.7.10^5 S/N=10.3
2.5 ppm I=2.3.10^5 S/N=14.9
Cookie
Chocolate
Jam
Compote
Almond GNLDFVQPPR
Almond prot; 0 mg/kg
571.8> 596.4
571.8> 743.4
Sensitivity
5 ppm I=4.1.10^5 S/N=22.9
2.5 ppm I=1.2.10^5 S/N=11.9
2.5 ppm I=9.1.10^5 S/N=34.7
2.5 ppm I=2.8.10^5 S/N=19.7
5 ppm I=3.8.10^5 S/N=22.0
2.5 ppm I=1.2.10^5 S/N=15.5
2.5 ppm I=8.7.10^5 S/N=75.4
2.5 ppm I=2.4.10^5 S/N=23.2

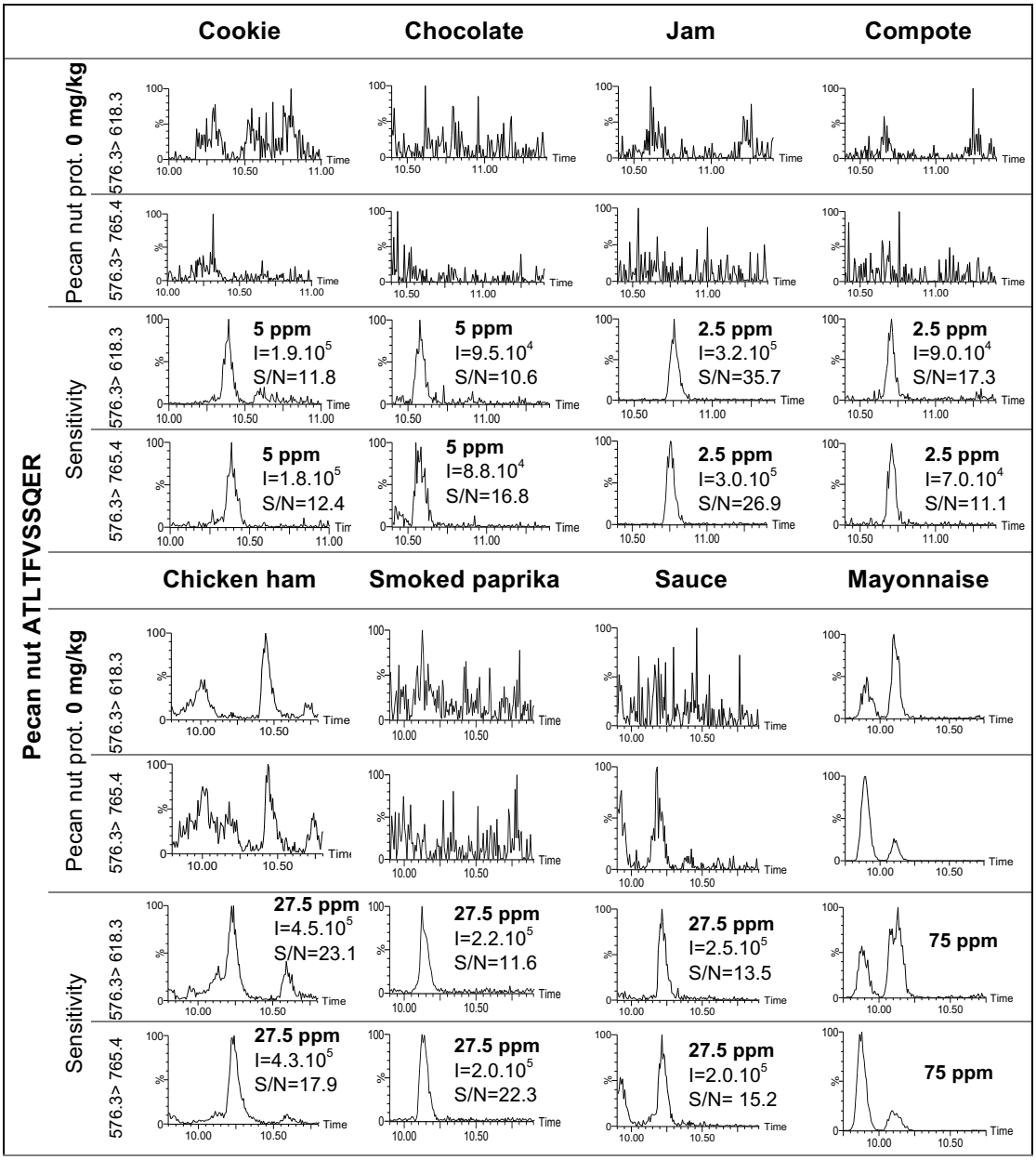

Chicken ham
Smoked paprika
Sauce
Mayonnaise
Almond GNLDFVQPPR
Almond prot; 0 mg/kg
571.8> 596.4
571.8> 743.4
Sensitivity
5 ppm I=5.3.10^5 S/N=14.4
2.5 ppm I=3.2.10^5 S/N=22.6
2.5 ppm I=2.0.10^5 S/N=14.8
2.5 ppm I=9.1.10^4 S/N=12.0
5 ppm I=4.8.10^5 S/N=9.1
2.5 ppm I=2.1.10^5 S/N=10.4
2.5 ppm I=1.6.10^5 S/N=10.7
2.5 ppm I=8.4.10^4 S/N=7.7
Cookie
Chocolate
Jam
Compote
Almond ALPDEVLANAYQISR
Almond prot. 0 mg/kg
830.4> 1035.6
830.4> 922.5
5 ppm I=1.7.10^5 S/N=7.6
2.5 ppm I=1.2.10^5 S/N=10.1
2.5 ppm I=5.8.10^5 S/N=140.3
15 ppm I=3.6.10^4 S/N=7.3
5 ppm I=1.6.10^5 S/N=15.0
2.5 ppm I=8.5.10^4 S/N=13.9
2.5 ppm I=5.8.10^5 S/N=70.9
15 ppm I=3.4.10^4 S/N=10.4
2.5 ppm I=3.2.10^5 S/N=25.9
2.5 ppm I=2.3.10^5 S/N=33.4
15 ppm I=5.4.10^4 S/N=12.9
27.5 ppm I=6.4.10^4 S/N=13.1
2.5 ppm I=3.0.10^5 S/N=21.5
2.5 ppm I=2.1.10^5 S/N=33.8
15 ppm I=5.0.10^4 S/N=3.2
27.5 ppm I=5.4.10^4 S/N=11.4
Time

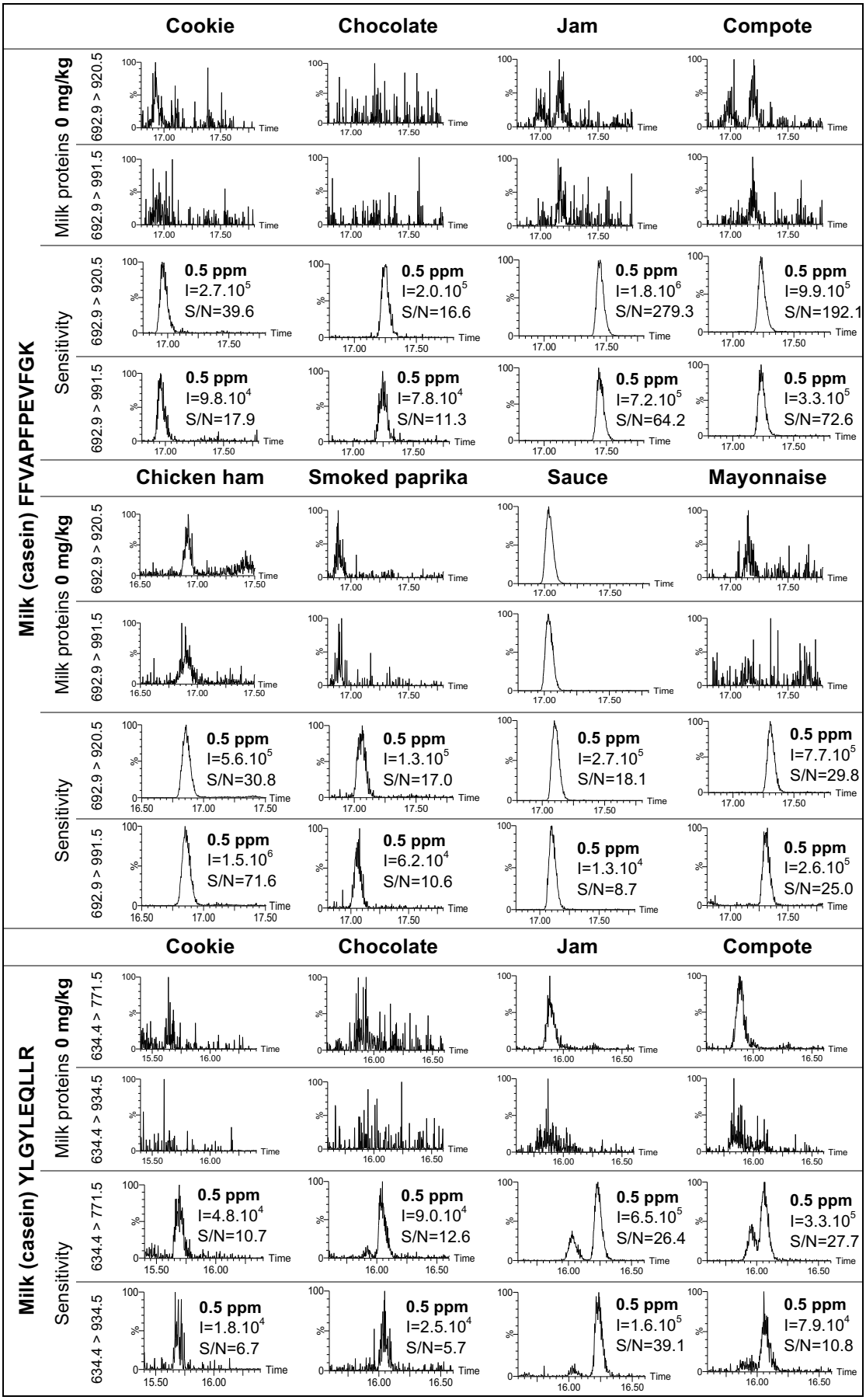

Cookie
Chocolate
Jam
Compote
Almond QETIALSSSQQR
Almond prot. 0 mg/kg
674.3> 876.5
674.3>692.3
Sensitivity
2.5 ppm I=2.4.10^5 S/N=14.3
2.5 ppm I=9.2.10^4 S/N=20.3
2.5 ppm I=4.6.10^5 S/N=37.4
2.5 ppm I=1.8.10^5 S/N=15.3
2.5 ppm I=2.3.10^5 S/N=14.8
2.5 ppm I=9.2.10^4 S/N=8.3
2.5 ppm I=4.2.10^5 S/N=100.4
2.5 ppm I=1.4.10^5 S/N=20.1
Chicken ham
Smoked paprika
Sauce
Mayonnaise
2.5 ppm I=2.4.10^5 S/N=17.7
5 ppm I=2.0.10^5 S/N=11.4
2.5 ppm I=9.9.10^4 S/N= 15.0
5 ppm I=8.0.10^4 S/N=13.9
2.5 ppm I=2.0.10^5 S/N=11.8
5 ppm I=1.2.10^5 S/N=15.0
2.5 ppm I=9.7.10^4 S/N=10.9
5 ppm I=7.8.10^4 S/N=26.7
Cookie
Chocolate
Jam
Compote
Almond TDENGFTNTLAGR
Almond prot. 0 mg/kg
698.3> 732.4
698.3> 879.5
Sensitivity
5 ppm I=1.3.10^5 S/N=16.2
5 ppm I=7.6.10^4 S/N=13.1
2.5 ppm I=2.0.10^5 S/N=17.7
7.5 ppm I=1.2.10^5 S/N=19.0
5 ppm I=3.4.10^4 S/N=4.6
5 ppm I=2.7.10^4 S/N=3.6
2.5 ppm I=9.0.10^4 S/N=14.5
7.5 ppm I=4.8.10^4 S/N=8.3

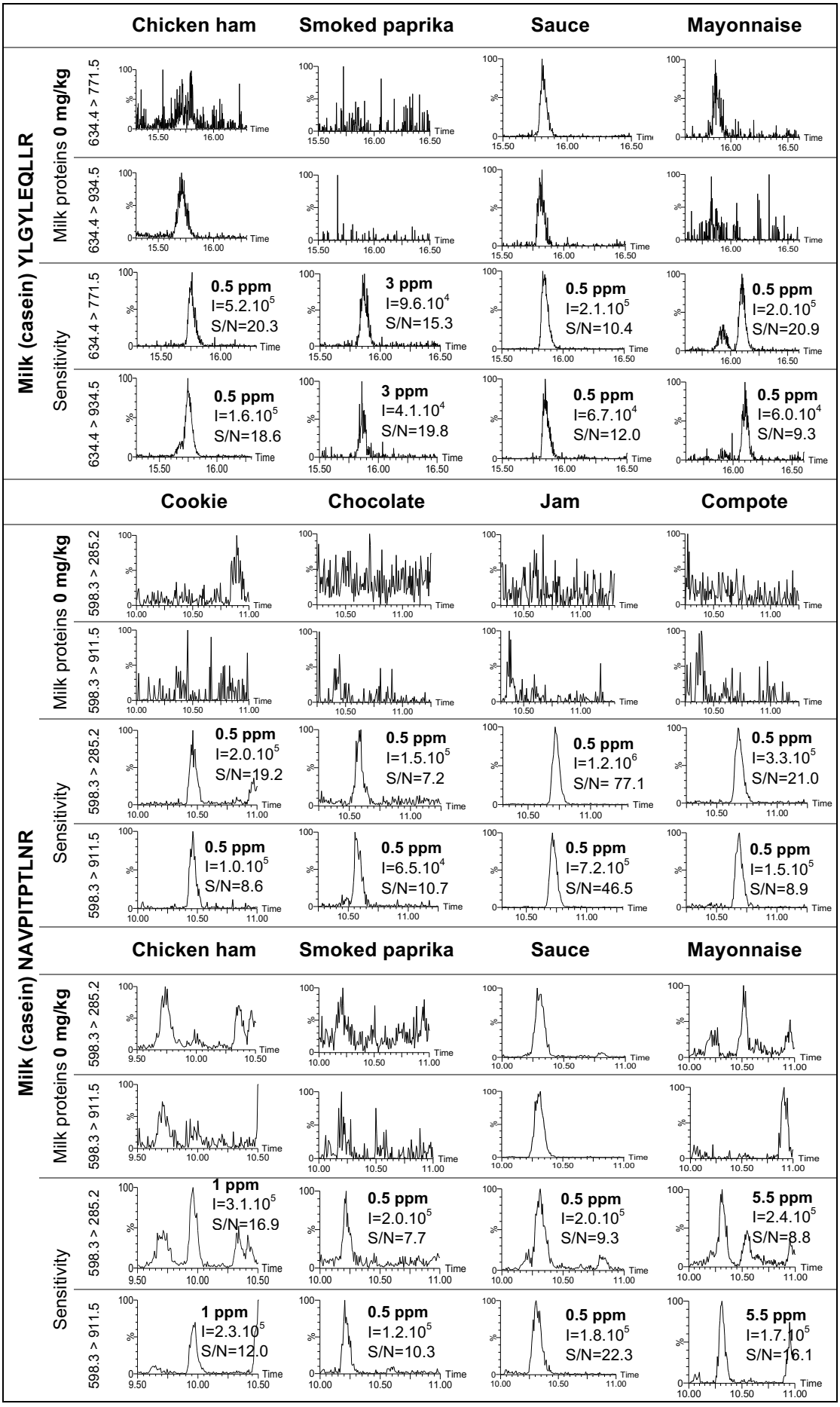

Chicken ham
Smoked paprika
Sauce
Mayonnaise
Almond TDENGFTNTLAGR
Almond prot. 0 mg/kg
698.3> 732.4
698.3> 879.5
Sensitivity
7.5 ppm I=1.6.10^5 S/N=13.1
15 ppm I=1.9.10^5 S/N=12.2
15 ppm I=1.4.10^5 S/N=11.4
52.5 ppm I=5.0.10^5 S/N=7.3
7.5 ppm I=5.3.10^4 S/N=8.0
15 ppm I=4.6.10^4 S/N=3.8
15 ppm I=7.0.10^4 S/N=6.3
52.5 ppm I=2.0.10^5 S/N=17.7
Cookie
Chocolate
Jam
Compote
Walnut GEEMEEMVQSAR
Walnut prot. 0 mg/kg
698.3> 820.4
689.3>949.4
2.5 ppm I=1.1.10^5 S/N=14.5
5 ppm I=4.1.10^4 S/N=7.7
2.5 ppm I=3.5.10^5 S/N= 27.9
2.5 ppm I=7.0.10^4 S/N=11.9
2.5 ppm I=9.3.10^4 S/N=16.0
5 ppm I=3.5.10^4 S/N=13.8
2.5 ppm I=3.3.10^5 S/N=67.9
2.5 ppm I=4.4.10^4 S/N=7.5
7.5 ppm I=1.6.10^5 S/N=15.9
7.5 ppm I=1.2.10^5 S/N=10.5
5 ppm I=1.0.10^5 S/N=12.4
2.5 ppm I=8.3.10^4 S/N=14.1
7.5 ppm I=5.3.10^4 S/N=8.0
7.5 ppm I=1.1.10^5 S/N=15.0
5 ppm I=9.3.10^4 S/N=13.7
2.5 ppm I=7.9.10^4 S/N=13.9
Time

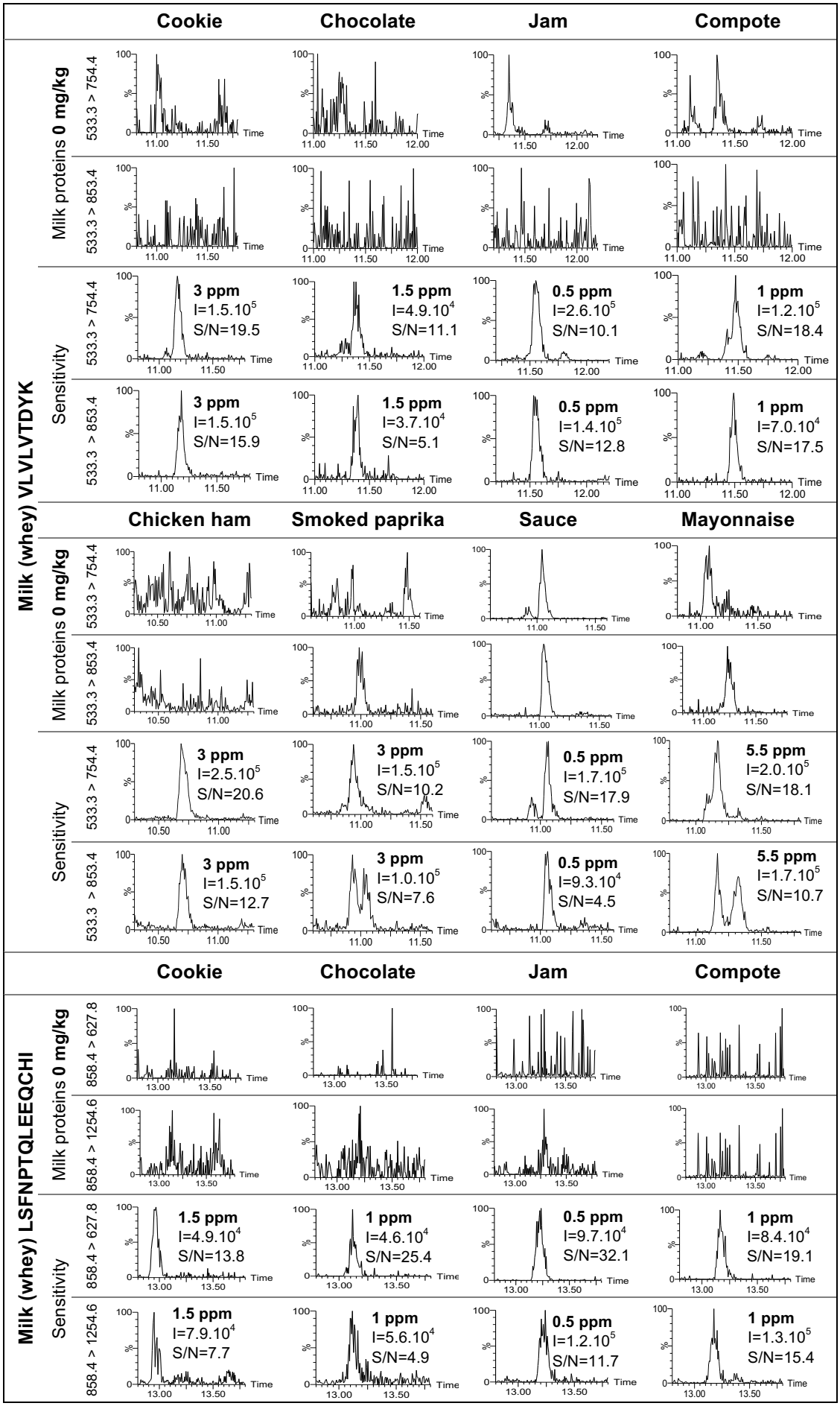

Cookie
Chocolate
Jam
Compote
Walnut ATLTLVSQETR
Walnut prot. 0 mg/kg
609.8> 620.3
609.8> 832.5
Sensitivity
15 ppm I=3.9.10^5 S/N=37.3
2.5 ppm I=7.1.10^4 S/N=12.7
2.5 ppm I=3.7.10^5 S/N=19.0
2.5 ppm I=1.2.10^5 S/N=16.2
15 ppm I=3.5.10^5 S/N=28.3
2.5 ppm I=2.8.10^4 S/N=6.6
2.5 ppm I=1.6.10^5 S/N=22.2
2.5 ppm I=5.7.10^4 S/N=7.4
Chicken ham
Smoked paprika
Sauce
Mayonnaise
15 ppm I=3.1.10^5 S/N=12.3
15 ppm I=2.2.10^5 S/N=11.5
15 ppm I=4.0.10^5 S/N= 22.1
15 ppm I=1.0.10^5 S/N=14.8
15 ppm I=1.7.10^5 S/N=15.0
15 ppm I=1.1.10^5 S/N=8.1
15 ppm I=2.3.10^5 S/N=11.8
15 ppm I=9.0.10^4 S/N=8.4
Pecan nut LVGFGGINGK
Pecan nut prot. 0
452.8> 692.4
452.8> 488.3
5 ppm I=6.3.10^5 S/N=19.2
2.5 ppm I=2.8.10^5 S/N=24.2
2.5 ppm I=1.3.10^6 S/N=57.9
5 ppm I=6.9.10^5 S/N=18.3
5 ppm I=1.3.10^5 S/N=8.4
2.5 ppm I=6.2.10^4 S/N=5.4
2.5 ppm I=2.5.10^5 S/N=26.5
5 ppm I=9.7.10^4 S/N=11.7

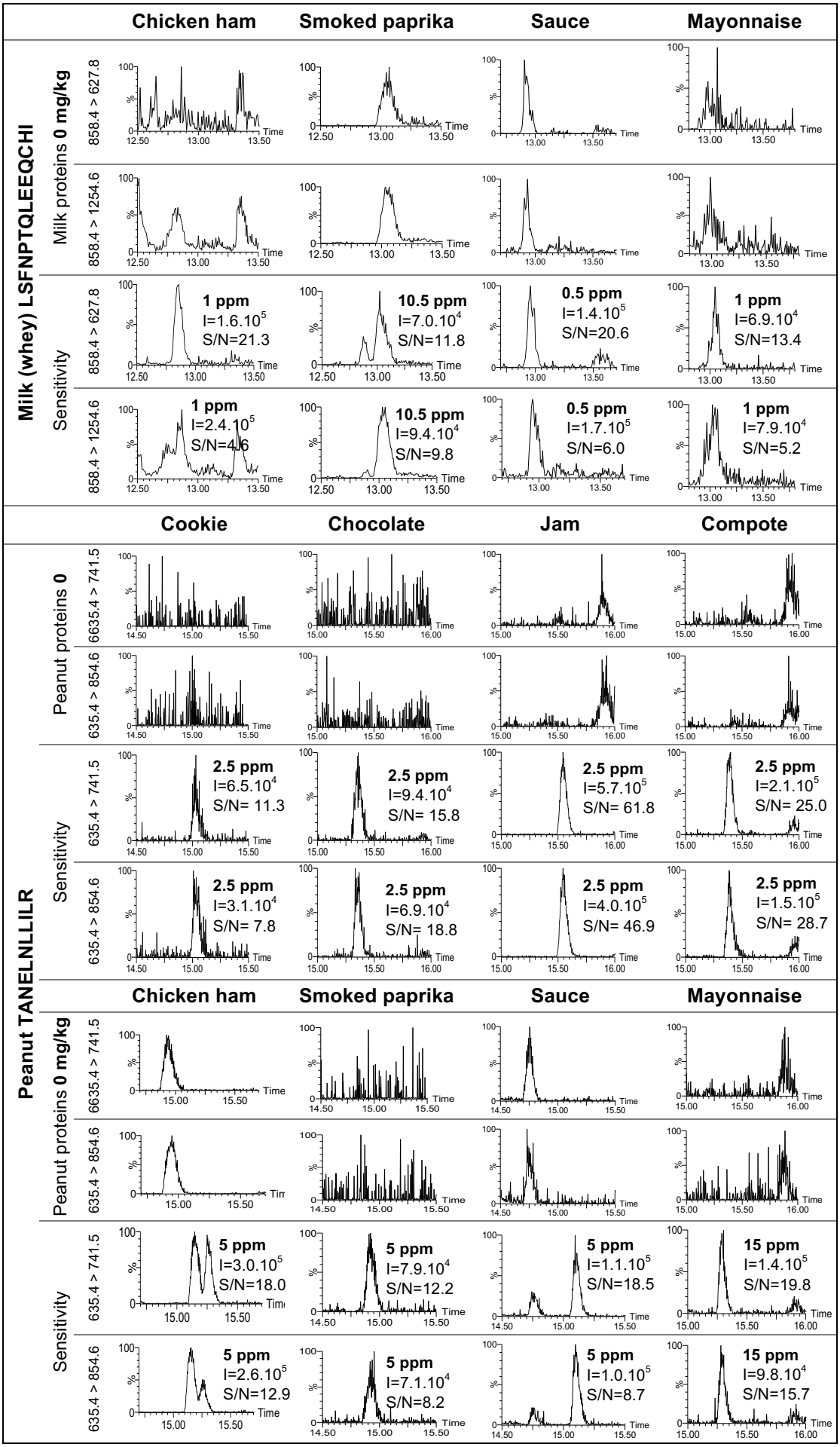

Chicken ham
Smoked paprika
Sauce
Mayonnaise
Pecan nut LVGFGGINGK
Pecan nut prot. 0
452.8> 692.4
452.8> 488.3
Sensitivity
5 ppm I=5.3.10^5 S/N=24.3
2.5 ppm I=2.7.10^5 S/N=15.0
5 ppm I=3.8.10^5 S/N=18.1
7.5 ppm I=3.7.10^5 S/N=31.8
5 ppm I=8.3.10^4 S/N=5.0
2.5 ppm I=6.8.10^4 S/N=3.5
5 ppm I=5.6.10^4 S/N=3.8
7.5 ppm I=5.8.10^4 S/N=4.2
Cookie
Chocolate
Jam
Compote
Pecan nut NFLAGQNNINQLER
Pecan nut prot. 0 mg/kg
582.0> 772.4
582.0> 659.7
7.5 ppm I=1.1.10^5 S/N=12.8
5 ppm I=6.7.10^4 S/N=18.0
2.5 ppm I=4.3.10^5 S/N=83.8
2.5 ppm I=1.5.10^5 S/N=15.0
7.5 ppm I=9.2.10^4 S/N=20.6
5 ppm I=6.4.10^4 S/N=14.7
2.5 ppm I=3.2.10^5 S/N= 30.7
2.5 ppm I=1.2.10^5 S/N=17.8
Chicken ham
Smoked paprika
Sauce
Mayonnaise
Pecan nut prot. 0 mg/kg
582.0> 772.4
582.0> 659.7
7.5 ppm I=3.0.10^5 S/N=16.7
15 ppm I=3.3.10^5 S/N=15.4
15 ppm I=1.0.10^5 S/N=11.2
5 ppm I=7.6.10^4 S/N=10.8
7.5 ppm I=2.8.10^5 S/N=13.0
15 ppm I=2.5.10^5 S/N=12.2
15 ppm I=7.3.10^4 S/N=6.0
5 ppm I=7.1.10^4 S/N=7.1
Time

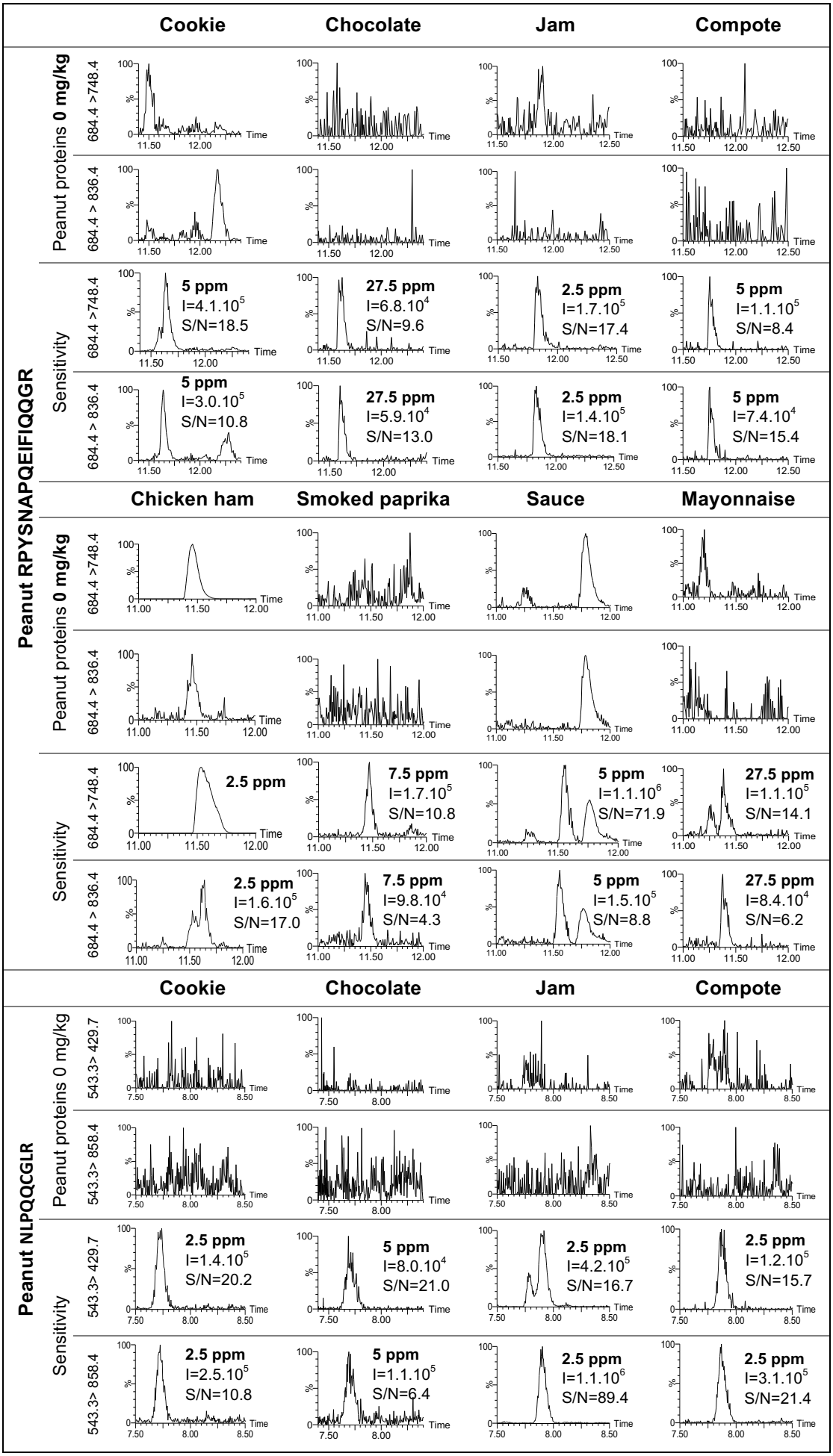

Pecan nut ATLTFVSSQER
Cookie
Chocolate
Jam
Compote
Pecan nut prot. 0 mg/kg
576.3> 618.3
576.3> 765.4
Sensitivity
5 ppm
I=1.9.10^5
S/N=11.8
5 ppm
I=9.5.10^4
S/N=10.6
2.5 ppm
I=3.2.10^5
S/N=35.7
2.5 ppm
I=9.0.10^4
S/N=17.3
5 ppm
I=1.8.10^5
S/N=12.4
5 ppm
I=8.8.10^4
S/N=16.8
2.5 ppm
I=3.0.10^5
S/N=26.9
2.5 ppm
I=7.0.10^4
S/N=11.1
Chicken ham
Smoked paprika
Sauce
Mayonnaise
27.5 ppm
I=4.5.10^5
S/N=23.1
27.5 ppm
I=2.2.10^5
S/N=11.6
27.5 ppm
I=2.5.10^5
S/N=13.5
75 ppm
27.5 ppm
I=4.3.10^5
S/N=17.9
27.5 ppm
I=2.0.10^5
S/N=22.3
27.5 ppm
I=2.0.10^5
S/N= 15.2
75 ppm
Time
%

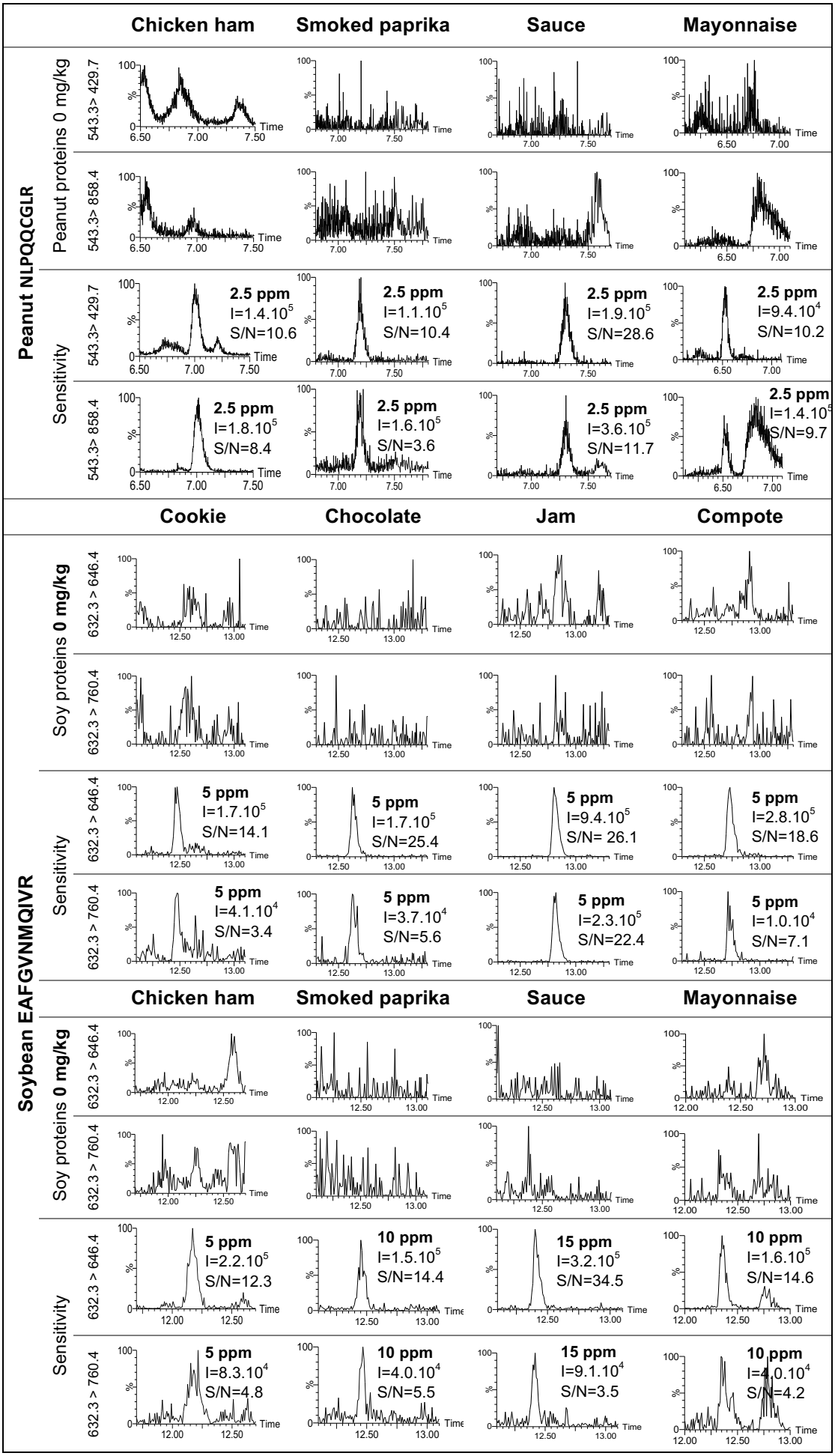

Cookie
Chocolate
Jam
Compote
Milk (casein) FFVAPFPEVFGK
Milk proteins 0 mg/kg
692.9 > 920.5
692.9 > 991.5
Sensitivity
0.5 ppm
I=2.7.10^5
S/N=39.6
0.5 ppm
I=2.0.10^5
S/N=16.6
0.5 ppm
I=1.8.10^6
S/N=279.3
0.5 ppm
I=9.9.10^5
S/N=192.1
0.5 ppm
I=9.8.10^4
S/N=17.9
0.5 ppm
I=7.8.10^4
S/N=11.3
0.5 ppm
I=7.2.10^5
S/N=64.2
0.5 ppm
I=3.3.10^5
S/N=72.6
Chicken ham
Smoked paprika
Sauce
Mayonnaise
0.5 ppm
I=5.6.10^5
S/N=30.8
0.5 ppm
I=1.3.10^5
S/N=17.0
0.5 ppm
I=2.7.10^5
S/N=18.1
0.5 ppm
I=7.7.10^5
S/N=29.8
0.5 ppm
I=1.5.10^6
S/N=71.6
0.5 ppm
I=6.2.10^4
S/N=10.6
0.5 ppm
I=1.3.10^4
S/N=8.7
0.5 ppm
I=2.6.10^5
S/N=25.0
Milk (casein) YLGYLEQLLR
Milk proteins 0 mg/kg
634.4 > 771.5
634.4 > 934.5
Sensitivity
0.5 ppm
I=4.8.10^4
S/N=10.7
0.5 ppm
I=9.0.10^4
S/N=12.6
0.5 ppm
I=6.5.10^5
S/N=26.4
0.5 ppm
I=3.3.10^5
S/N=27.7
0.5 ppm
I=1.8.10^4
S/N=6.7
0.5 ppm
I=2.5.10^4
S/N=5.7
0.5 ppm
I=1.6.10^5
S/N=39.1
0.5 ppm
I=7.9.10^4
S/N=10.8

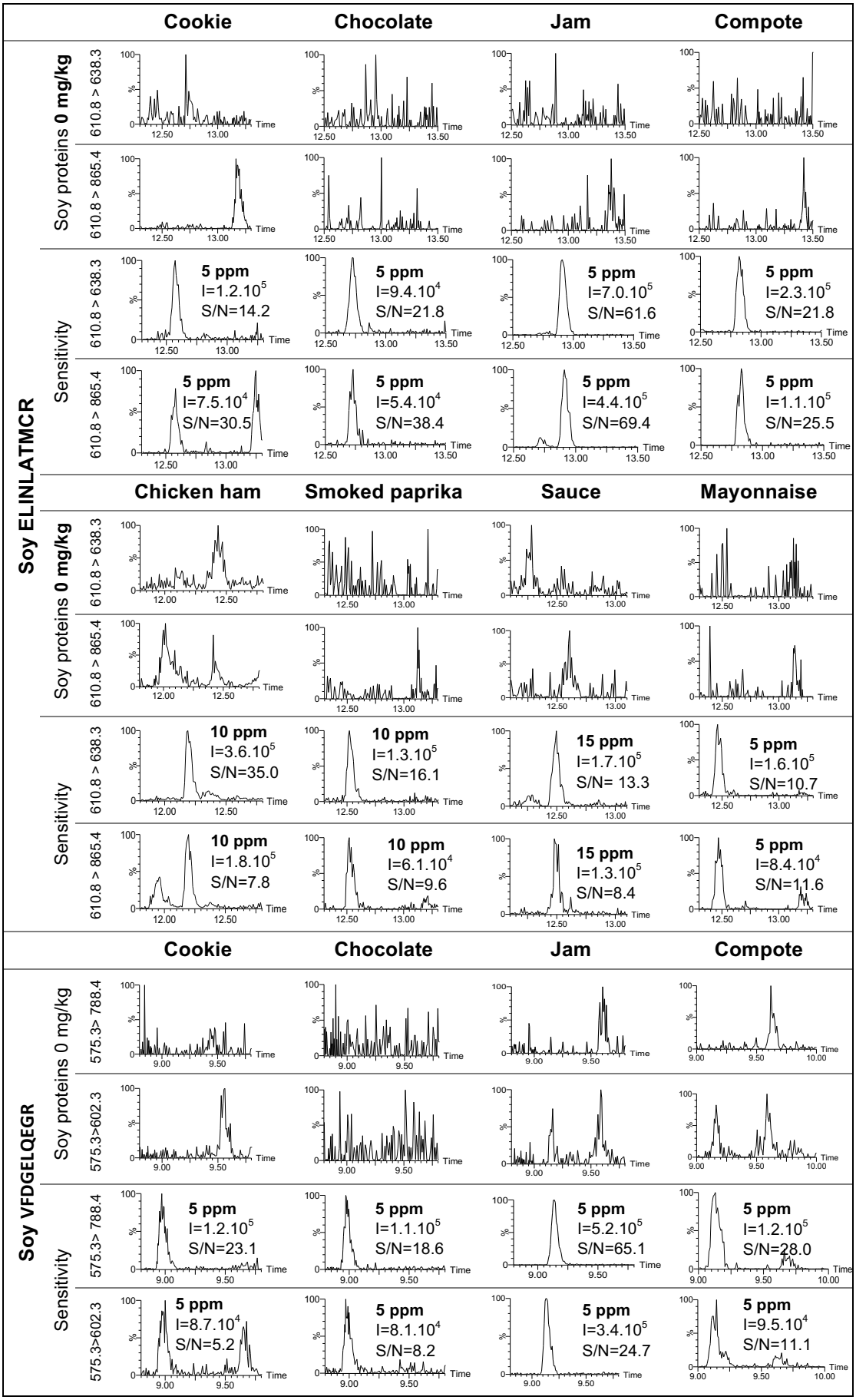

Chicken ham
Smoked paprika
Sauce
Mayonnaise
Milk (casein) YLGYLEQLLR
Milk proteins 0 mg/kg
634.4 > 771.5
634.4 > 934.5
Sensitivity
0.5 ppm I=5.2.10^5 S/N=20.3
3 ppm I=9.6.10^4 S/N=15.3
0.5 ppm I=2.1.10^5 S/N=10.4
0.5 ppm I=2.0.10^5 S/N=20.9
0.5 ppm I=1.6.10^5 S/N=18.6
3 ppm I=4.1.10^4 S/N=19.8
0.5 ppm I=6.7.10^4 S/N=12.0
0.5 ppm I=6.0.10^4 S/N=9.3
Cookie
Chocolate
Jam
Compote
Milk (casein) NAVPITPTLNR
598.3 > 285.2
598.3 > 911.5
0.5 ppm I=2.0.10^5 S/N=19.2
0.5 ppm I=1.5.10^5 S/N=7.2
0.5 ppm I=1.2.10^6 S/N= 77.1
0.5 ppm I=3.3.10^5 S/N=21.0
0.5 ppm I=1.0.10^5 S/N=8.6
0.5 ppm I=6.5.10^4 S/N=10.7
0.5 ppm I=7.2.10^5 S/N=46.5
0.5 ppm I=1.5.10^5 S/N=8.9
1 ppm I=3.1.10^5 S/N=16.9
0.5 ppm I=2.0.10^5 S/N=7.7
0.5 ppm I=2.0.10^5 S/N=9.3
5.5 ppm I=2.4.10^5 S/N=8.8
1 ppm I=2.3.10^5 S/N=12.0
0.5 ppm I=1.2.10^5 S/N=10.3
0.5 ppm I=1.8.10^5 S/N=22.3
5.5 ppm I=1.7.10^5 S/N=16.1
Time

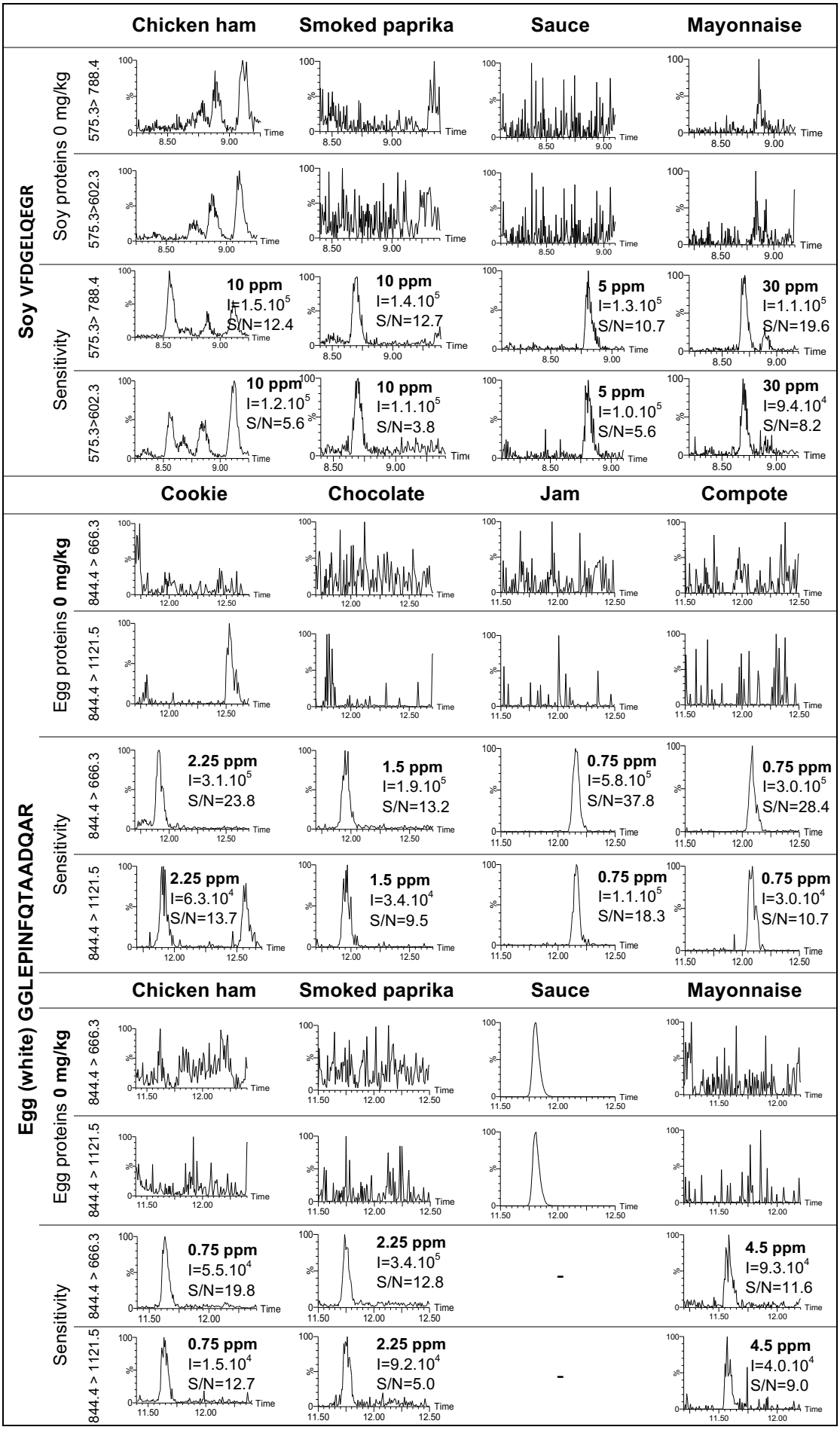

Cookie
Chocolate
Jam
Compote
Milk (whey) VLVLVTDYK
Milk proteins 0 mg/kg
533.3 > 754.4
533.3 > 853.4
Sensitivity
3 ppm I=1.5.10^5 S/N=19.5
1.5 ppm I=4.9.10^4 S/N=11.1
0.5 ppm I=2.6.10^5 S/N=10.1
1 ppm I=1.2.10^5 S/N=18.4
3 ppm I=1.5.10^5 S/N=15.9
1.5 ppm I=3.7.10^4 S/N=5.1
0.5 ppm I=1.4.10^5 S/N=12.8
1 ppm I=7.0.10^4 S/N=17.5
Chicken ham
Smoked paprika
Sauce
Mayonnaise
3 ppm I=2.5.10^5 S/N=20.6
3 ppm I=1.5.10^5 S/N=10.2
0.5 ppm I=1.7.10^5 S/N=17.9
5.5 ppm I=2.0.10^5 S/N=18.1
3 ppm I=1.5.10^5 S/N=12.7
3 ppm I=1.0.10^5 S/N=7.6
0.5 ppm I=9.3.10^4 S/N=4.5
5.5 ppm I=1.7.10^5 S/N=10.7
Cookie
Chocolate
Jam
Compote
Milk (whey) LSFNPTQLEEQCHI
Milk proteins 0 mg/kg
858.4 > 627.8
858.4 > 1254.6
Sensitivity
1.5 ppm I=4.9.10^4 S/N=13.8
1 ppm I=4.6.10^4 S/N=25.4
0.5 ppm I=9.7.10^4 S/N=32.1
1 ppm I=8.4.10^4 S/N=19.1
1.5 ppm I=7.9.10^4 S/N=7.7
1 ppm I=5.6.10^4 S/N=4.9
0.5 ppm I=1.2.10^5 S/N=11.7
1 ppm I=1.3.10^5 S/N=15.4
Time

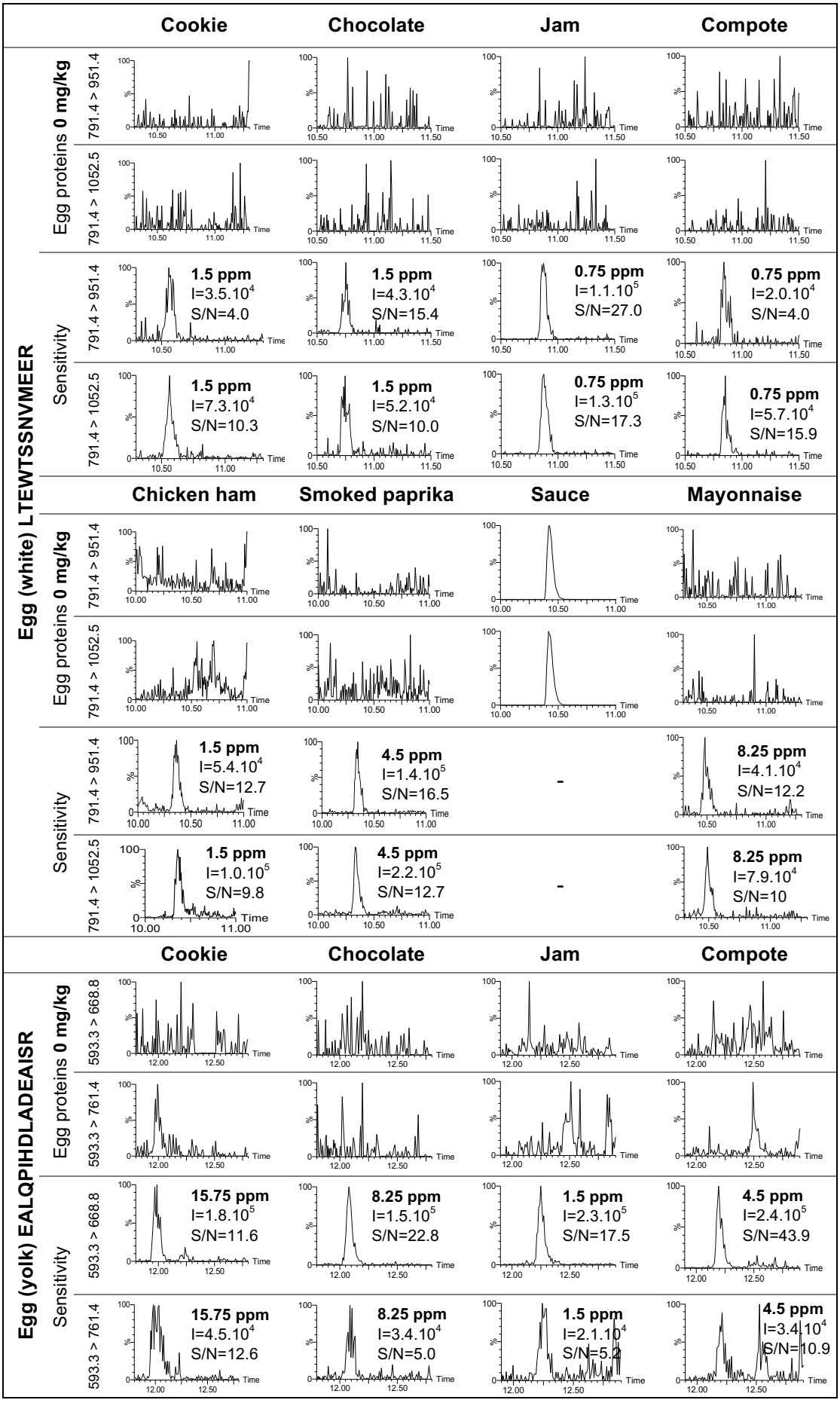

Chicken ham
Smoked paprika
Sauce
Mayonnaise
Milk (whey) LSFNPTQLEEQCHI
Milk proteins 0 mg/kg
858.4 > 627.8
858.4 > 1254.6
Sensitivity
1 ppm I=1.6.10^5 S/N=21.3
10.5 ppm I=7.0.10^4 S/N=11.8
0.5 ppm I=1.4.10^5 S/N=20.6
1 ppm I=6.9.10^4 S/N=13.4
1 ppm I=2.4.10^5 S/N=4.6
10.5 ppm I=9.4.10^4 S/N=9.8
0.5 ppm I=1.7.10^5 S/N=6.0
1 ppm I=7.9.10^4 S/N=5.2
Cookie
Chocolate
Jam
Compote
Peanut TANELNLLILR
Peanut proteins 0
6635.4 > 741.5
635.4 > 854.6
2.5 ppm I=6.5.10^4 S/N= 11.3
2.5 ppm I=9.4.10^4 S/N= 15.8
2.5 ppm I=5.7.10^5 S/N= 61.8
2.5 ppm I=2.1.10^5 S/N= 25.0
2.5 ppm I=3.1.10^4 S/N= 7.8
2.5 ppm I=6.9.10^4 S/N= 18.8
2.5 ppm I=4.0.10^5 S/N= 46.9
2.5 ppm I=1.5.10^5 S/N= 28.7
Peanut proteins 0 mg/kg
5 ppm I=3.0.10^5 S/N=18.0
5 ppm I=7.9.10^4 S/N=12.2
5 ppm I=1.1.10^5 S/N=18.5
15 ppm I=1.4.10^5 S/N=19.8
5 ppm I=2.6.10^5 S/N=12.9
5 ppm I=7.1.10^4 S/N=8.2
5 ppm I=1.0.10^5 S/N=8.7
15 ppm I=9.8.10^4 S/N=15.7

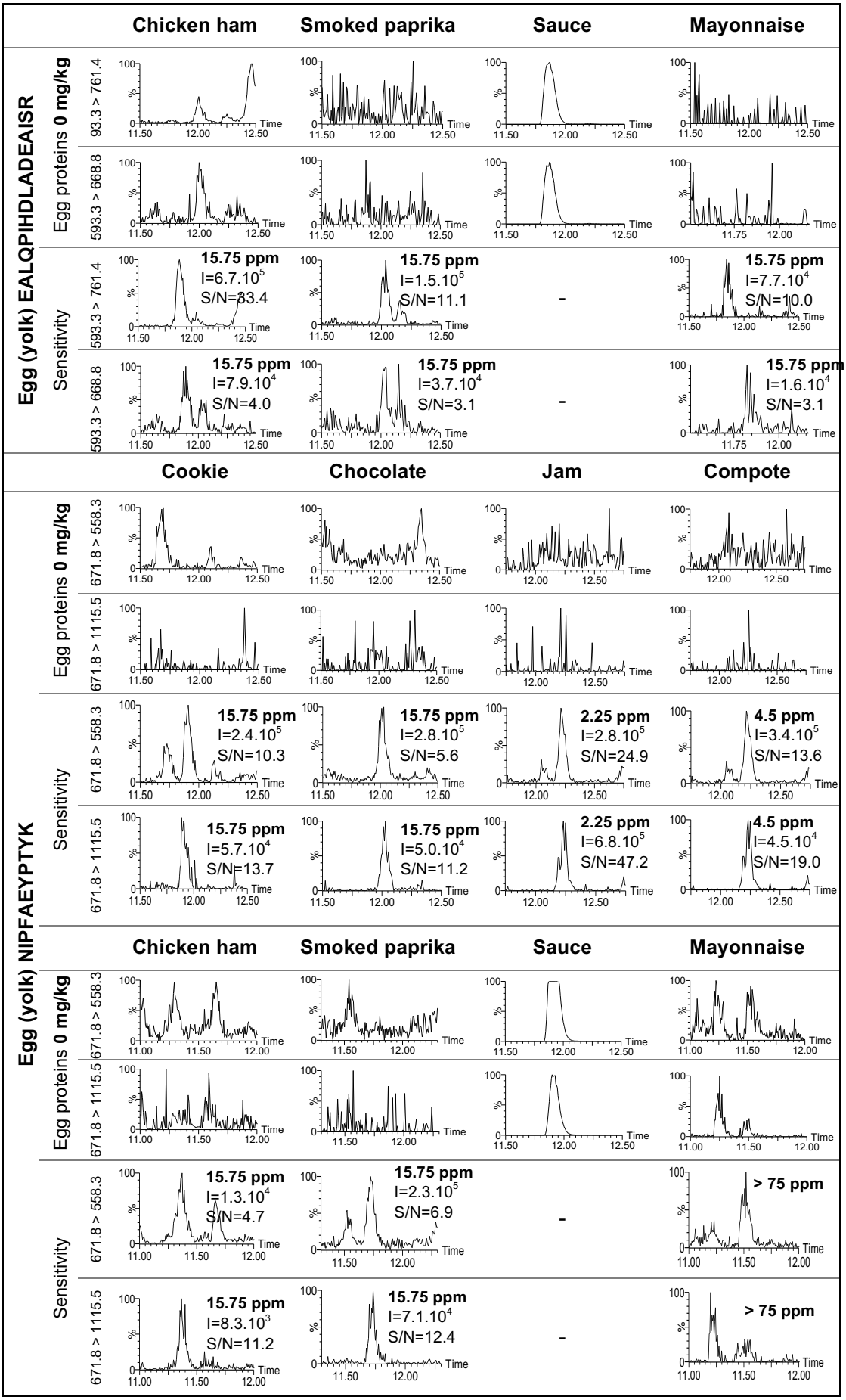

Cookie
Chocolate
Jam
Compote
Peanut RPYSNAPQEIFIQQGR
Peanut proteins 0 mg/kg
684.4 >748.4
684.4 > 836.4
Sensitivity
5 ppm I=4.1.10^5 S/N=18.5
27.5 ppm I=6.8.10^4 S/N=9.6
2.5 ppm I=1.7.10^5 S/N=17.4
5 ppm I=1.1.10^5 S/N=8.4
5 ppm I=3.0.10^5 S/N=10.8
27.5 ppm I=5.9.10^4 S/N=13.0
2.5 ppm I=1.4.10^5 S/N=18.1
5 ppm I=7.4.10^4 S/N=15.4
Chicken ham
Smoked paprika
Sauce
Mayonnaise
2.5 ppm
7.5 ppm I=1.7.10^5 S/N=10.8
5 ppm I=1.1.10^6 S/N=71.9
27.5 ppm I=1.1.10^5 S/N=14.1
2.5 ppm I=1.6.10^5 S/N=17.0
7.5 ppm I=9.8.10^4 S/N=4.3
5 ppm I=1.5.10^5 S/N=8.8
27.5 ppm I=8.4.10^4 S/N=6.2
Peanut NLPQQCGLR
Peanut proteins 0 mg/kg
543.3> 429.7
543.3> 858.4
2.5 ppm I=1.4.10^5 S/N=20.2
5 ppm I=8.0.10^4 S/N=21.0
2.5 ppm I=4.2.10^5 S/N=16.7
2.5 ppm I=1.2.10^5 S/N=15.7
2.5 ppm I=2.5.10^5 S/N=10.8
5 ppm I=1.1.10^5 S/N=6.4
2.5 ppm I=1.1.10^6 S/N=89.4
2.5 ppm I=3.1.10^5 S/N=21.4

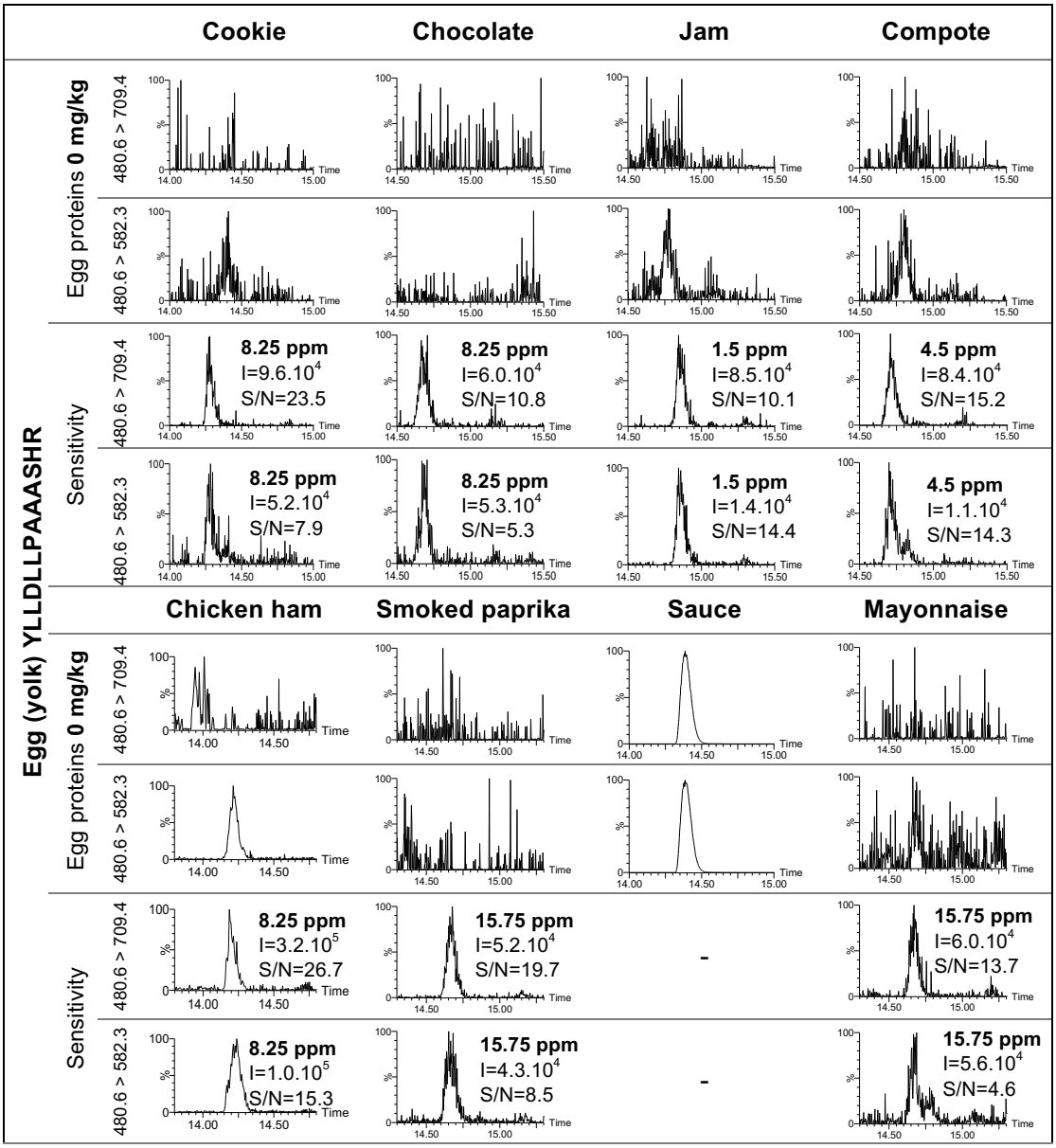

Chicken ham
Smoked paprika
Sauce
Mayonnaise
Peanut NLPQQCGLR
Peanut proteins 0 mg/kg
543.3> 429.7
543.3> 858.4
Sensitivity
2.5 ppm
I=1.4.10^5
S/N=10.6
2.5 ppm
I=1.1.10^5
S/N=10.4
2.5 ppm
I=1.9.10^5
S/N=28.6
2.5 ppm
I=9.4.10^4
S/N=10.2
2.5 ppm
I=1.8.10^5
S/N=8.4
2.5 ppm
I=1.6.10^5
S/N=3.6
2.5 ppm
I=3.6.10^5
S/N=11.7
2.5 ppm
I=1.4.10^5
S/N=9.7
Cookie
Chocolate
Jam
Compote
Soybean EAFGVNMQIVR
Soy proteins 0 mg/kg
632.3 > 646.4
632.3 > 760.4
Sensitivity
5 ppm
I=1.7.10^5
S/N=14.1
5 ppm
I=1.7.10^5
S/N=25.4
5 ppm
I=9.4.10^5
S/N= 26.1
5 ppm
I=2.8.10^5
S/N=18.6
5 ppm
I=4.1.10^4
S/N=3.4
5 ppm
I=3.7.10^4
S/N=5.6
5 ppm
I=2.3.10^5
S/N=22.4
5 ppm
I=1.0.10^4
S/N=7.1
Chicken ham
Smoked paprika
Sauce
Mayonnaise
Soy proteins 0 mg/kg
632.3 > 646.4
632.3 > 760.4
Sensitivity
5 ppm
I=2.2.10^5
S/N=12.3
10 ppm
I=1.5.10^5
S/N=14.4
15 ppm
I=3.2.10^5
S/N=34.5
10 ppm
I=1.6.10^5
S/N=14.6
5 ppm
I=8.3.10^4
S/N=4.8
10 ppm
I=4.0.10^4
S/N=5.5
15 ppm
I=9.1.10^4
S/N=3.5
10 ppm
I=4.0.10^4
S/N=4.2


Cookie
Chocolate
Jam
Compote
Soy ELINLATMCR
Soy proteins 0 mg/kg
610.8 > 638.3
610.8 > 865.4
Sensitivity
5 ppm I=1.2.10^5 S/N=14.2
5 ppm I=9.4.10^4 S/N=21.8
5 ppm I=7.0.10^5 S/N=61.6
5 ppm I=2.3.10^5 S/N=21.8
5 ppm I=7.5.10^4 S/N=30.5
5 ppm I=5.4.10^4 S/N=38.4
5 ppm I=4.4.10^5 S/N=69.4
5 ppm I=1.1.10^5 S/N=25.5
Chicken ham
Smoked paprika
Sauce
Mayonnaise
10 ppm I=3.6.10^5 S/N=35.0
10 ppm I=1.3.10^5 S/N=16.1
15 ppm I=1.7.10^5 S/N= 13.3
5 ppm I=1.6.10^5 S/N=10.7
10 ppm I=1.8.10^5 S/N=7.8
10 ppm I=6.1.10^4 S/N=9.6
15 ppm I=1.3.10^5 S/N=8.4
5 ppm I=8.4.10^4 S/N=11.6
Cookie
Chocolate
Jam
Compote
Soy VFDGELQEGR
Soy proteins 0 mg/kg
575.3> 788.4
575.3>602.3
Sensitivity
5 ppm I=1.2.10^5 S/N=23.1
5 ppm I=1.1.10^5 S/N=18.6
5 ppm I=5.2.10^5 S/N=65.1
5 ppm I=1.2.10^5 S/N=28.0
5 ppm I=8.7.10^4 S/N=5.2
5 ppm I=8.1.10^4 S/N=8.2
5 ppm I=3.4.10^5 S/N=24.7
5 ppm I=9.5.10^4 S/N=11.1

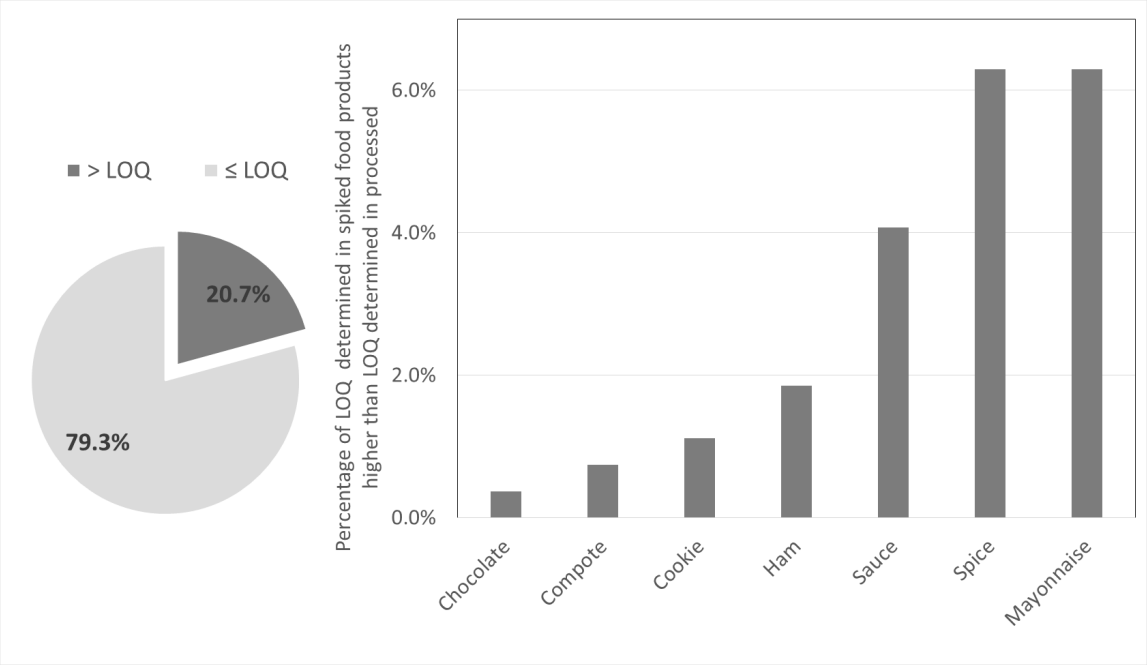

Chicken ham
Smoked paprika
Sauce
Mayonnaise
Soy VFDGELQEGR
Soy proteins 0 mg/kg
575.3> 788.4
575.3>602.3
Sensitivity
10 ppm I=1.5.10^5 S/N=12.4
10 ppm I=1.4.10^5 S/N=12.7
5 ppm I=1.3.10^5 S/N=10.7
30 ppm I=1.1.10^5 S/N=19.6
10 ppm I=1.2.10^5 S/N=5.6
10 ppm I=1.1.10^5 S/N=3.8
5 ppm I=1.0.10^5 S/N=5.6
30 ppm I=9.4.10^4 S/N=8.2
Cookie
Chocolate
Jam
Compote
Egg (white) GGLEPINFQTAADQAR
Egg proteins 0 mg/kg
844.4 > 666.3
844.4 > 1121.5
2.25 ppm I=3.1.10^5 S/N=23.8
1.5 ppm I=1.9.10^5 S/N=13.2
0.75 ppm I=5.8.10^5 S/N=37.8
0.75 ppm I=3.0.10^5 S/N=28.4
2.25 ppm I=6.3.10^4 S/N=13.7
1.5 ppm I=3.4.10^4 S/N=9.5
0.75 ppm I=1.1.10^5 S/N=18.3
0.75 ppm I=3.0.10^4 S/N=10.7
0.75 ppm I=5.5.10^4 S/N=19.8
2.25 ppm I=3.4.10^5 S/N=12.8
4.5 ppm I=9.3.10^4 S/N=11.6
0.75 ppm I=1.5.10^4 S/N=12.7
2.25 ppm I=9.2.10^4 S/N=5.0
4.5 ppm I=4.0.10^4 S/N=9.0
Time


Cookie
Chocolate
Jam
Compote
Egg (white) LTEWTSSNVMEER
Egg proteins 0 mg/kg
791.4 > 951.4
791.4 > 1052.5
Sensitivity
1.5 ppm I=3.5.10^4 S/N=4.0
1.5 ppm I=4.3.10^4 S/N=15.4
0.75 ppm I=1.1.10^5 S/N=27.0
0.75 ppm I=2.0.10^4 S/N=4.0
1.5 ppm I=7.3.10^4 S/N=10.3
1.5 ppm I=5.2.10^4 S/N=10.0
0.75 ppm I=1.3.10^5 S/N=17.3
0.75 ppm I=5.7.10^4 S/N=15.9
Chicken ham
Smoked paprika
Sauce
Mayonnaise
1.5 ppm I=5.4.10^4 S/N=12.7
4.5 ppm I=1.4.10^5 S/N=16.5
-
8.25 ppm I=4.1.10^4 S/N=12.2
1.5 ppm I=1.0.10^5 S/N=9.8
4.5 ppm I=2.2.10^5 S/N=12.7
-
8.25 ppm I=7.9.10^4 S/N=10
Cookie
Chocolate
Jam
Compote
Egg (yolk) EALQPIHDLADEAISR
Egg proteins 0 mg/kg
593.3 > 668.8
593.3 > 761.4
Sensitivity
15.75 ppm I=1.8.10^5 S/N=11.6
8.25 ppm I=1.5.10^5 S/N=22.8
1.5 ppm I=2.3.10^5 S/N=17.5
4.5 ppm I=2.4.10^5 S/N=43.9
15.75 ppm I=4.5.10^4 S/N=12.6
8.25 ppm I=3.4.10^4 S/N=5.0
1.5 ppm I=2.1.10^4 S/N=5.2
4.5 ppm I=3.4.10^4 S/N=10.9
Time

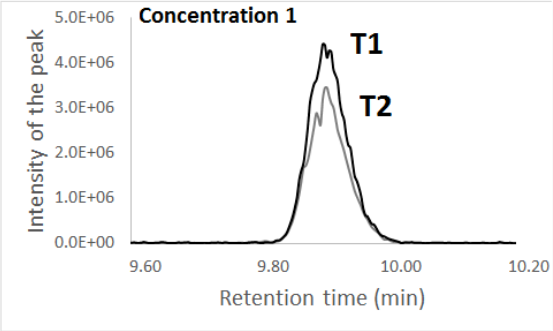

Chicken ham
Smoked paprika
Sauce
Mayonnaise
Egg (yolk) EALQPIHDLADEAISR
Egg proteins 0 mg/kg
93.3 > 761.4
593.3 > 668.8
Sensitivity
593.3 > 761.4
15.75 ppm
I=6.7.10^5
S/N=33.4
15.75 ppm
I=1.5.10^5
S/N=11.1
-
15.75 ppm
I=7.7.10^4
S/N=10.0
593.3 > 668.8
15.75 ppm
I=7.9.10^4
S/N=4.0
15.75 ppm
I=3.7.10^4
S/N=3.1
-
15.75 ppm
I=1.6.10^4
S/N=3.1
Cookie
Chocolate
Jam
Compote
Egg (yolk) NIPFAEYPTYK
Egg proteins 0 mg/kg
671.8 > 558.3
671.8 > 1115.5
Sensitivity
671.8 > 558.3
15.75 ppm
I=2.4.10^5
S/N=10.3
15.75 ppm
I=2.8.10^5
S/N=5.6
2.25 ppm
I=2.8.10^5
S/N=24.9
4.5 ppm
I=3.4.10^5
S/N=13.6
671.8 > 1115.5
15.75 ppm
I=5.7.10^4
S/N=13.7
15.75 ppm
I=5.0.10^4
S/N=11.2
2.25 ppm
I=6.8.10^5
S/N=47.2
4.5 ppm
I=4.5.10^4
S/N=19.0
Chicken ham
Smoked paprika
Sauce
Mayonnaise
Egg proteins 0 mg/kg
671.8 > 558.3
671.8 > 1115.5
Sensitivity
671.8 > 558.3
15.75 ppm
I=1.3.10^4
S/N=4.7
15.75 ppm
I=2.3.10^5
S/N=6.9
-
> 75 ppm
671.8 > 1115.5
15.75 ppm
I=8.3.10^3
S/N=11.2
15.75 ppm
I=7.1.10^4
S/N=12.4
-
> 75 ppm
Time
%
100
0
11.50
12.00
12.50
11.00
11.75

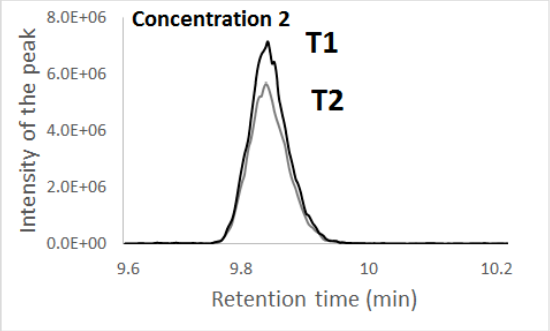

Egg (yolk) YLLDLLPAAASHR
Egg proteins 0 mg/kg
Sensitivity
480.6 > 709.4
480.6 > 582.3
Cookie
Chocolate
Jam
Compote
8.25 ppm I=9.6.10^4 S/N=23.5
8.25 ppm I=6.0.10^4 S/N=10.8
1.5 ppm I=8.5.10^4 S/N=10.1
4.5 ppm I=8.4.10^4 S/N=15.2
8.25 ppm I=5.2.10^4 S/N=7.9
8.25 ppm I=5.3.10^4 S/N=5.3
1.5 ppm I=1.4.10^4 S/N=14.4
4.5 ppm I=1.1.10^4 S/N=14.3
Chicken ham
Smoked paprika
Sauce
Mayonnaise
8.25 ppm I=3.2.10^5 S/N=26.7
15.75 ppm I=5.2.10^4 S/N=19.7
-
15.75 ppm I=6.0.10^4 S/N=13.7
8.25 ppm I=1.0.10^5 S/N=15.3
15.75 ppm I=4.3.10^4 S/N=8.5
-
15.75 ppm I=5.6.10^4 S/N=4.6
Time
%

***Supplementary material – Figure 2*:** (A) Percentage of LOQs determined in eight spiked matrices for 35 peptides (n=270 LOQs due to the exclusion of egg and milk peptides in sauce) and found to be lower than or equal to (79.3 %) or higher than (20.7%) the LOQ determined in processed matrices. (B) Percentage of LOQs (for each matrix) found to be higher than the LOQs determined in processed matrices.



Supplementary material - Figure 3: *Calculation of the ion ratio (%) and the relative ion intensity (%). The criteria of acceptation for the regulation 2002/657/EC (analysis of veterinary drug residues) and SANTE/11813/2017 (pesticides) are also presented.*





Ion ratio 1 (%) = Intensity T2 / Intensity T1 x 100

Ion ratio 2 (%) = Intensity T2 / Intensity T1 x 100

Relative ion intensity (%) = (|Ion ratio 2 - Ion ratio 1| x 100) / Ion ratio 1

Relative intensity (% of base peak)	CI-GC-MS, GC-MS^n LC-MS, LC-MS^n (relative)
> 50 %	± 20 %
> 20 % to 50 %	± 25 %
> 10 % to 20 %	± 30 %
≤ 10 %	± 50 %

Maximum permitted tolerances for relative ion intensities using a range of mass spectrometry techniques modified from 2002/657/EC

MS detector/Characteristics		Acquisition	Requirements for identification	
Resolution	Typical systems (examples)		minimum number of ions	other
Unit mass resolution	MS/MS triple quadrupole, ion trap, Q-trap, Q-TOF, Q-Orbitrap	selected or multiple reaction monitoring (SRM, MRM), mass resolution for precursor-ion isolation equal to or better than unit mass resolution	2 product ions	Ion ratio from sample extracts should be within **±30% (relative)** of average of calibration standards from same sequence

Relative ion intensity : 30%

Identification requirements for different MS techniques modified from SANTE/11813/2017

Supplementary material - Table 1: *Peak intensity, ion ratios between transitions, and relative ion intensities between ion ratios at two concentrations for RPYSNAPQEIFIQQGR (peanut) and ATLTFVSSQER (pecan nut). The relative ion intensitiy was calculated between the LOQ and 52.5 mg peanut or pecan nut.*

	RPYSNAPQEIFIQQGR (peanut)					ATLTFVSSQER (pecan nut)				
	Concentration (mg of proteins per kg)	Transition (T)	Peak intensity	Ion ratio (%)	relative ion intensities (%)	Concentration (mg of proteins per kg)	Transition (T)	Peak intensity	Ion ratio (%)	relative ion intensities (%)
Chocolate	27.5	T1	6.79E+04	87%	7.8%	5	T1	9.52E+04	92%	8.3%
		T2	5.94E+04				T2	8.79E+04		
	52.5	T1	1.23E+05	94%		52.5	T1	7.39E+05	85%	
		T2	1.16E+05				T2	6.26E+05		
Compote	5	T1	1.13E+05	65%	13.7%	2.5	T1	9.02E+04	78%	14.7%
		T2	7.37E+04				T2	7.03E+04		
	52.5	T1	8.82E+05	74%		52.5	T1	1.23E+06	89%	
		T2	6.54E+05				T2	1.10E+06		
Jam	2.5	T1	1.74E+05	79%	5.9%	2.5	T1	3.21E+05	94%	1.3%
		T2	1.38E+05				T2	3.01E+05		
	52.5	T1	9.11E+05	84%		52.5	T1	3.62E+06	93%	
		T2	7.65E+05				T2	3.35E+06		
Cookie	5	T1	4.80E+05	66%	11.7%	5	T1	1.89E+05	96%	2.2%
		T2	3.16E+05				T2	1.81E+05		
	52.5	T1	2.87E+06	74%		52.5	T1	1.26E+06	94%	
		T2	2.11E+06				T2	1.18E+06		
Spice	7.5	T1	1.71E+05	57%	19.6%	27.5	T1	2.21E+05	89%	2.5%
		T2	9.83E+04				T2	1.96E+05		
	52.5	T1	5.47E+05	69%		52.5	T1	4.88E+05	86%	
		T2	3.76E+05				T2	4.22E+05		
Ham	2.5	T1	1.27E+08	0%	965.7%	27.5	T1	4.47E+05	97%	10.1%
		T2	1.64E+05				T2	4.32E+05		
	52.5	T1	1.01E+08	1%		52.5	T1	1.04E+06	87%	
		T2	1.39E+06				T2	9.04E+05		
Mayonnaise	27.5	T1	1.05E+05	80%	17.4%	2.5	T1	5.17E+05	57%	36.8%
		T2	8.43E+04				T2	2.95E+05		
	52.5	T1	3.49E+05	94%		52.5	T1	5.06E+05	78%	
		T2	3.29E+05				T2	3.95E+05		
Sauce	5	T1	1.69E+05	76%	18.4%	27.5	T1	2.12E+05	92%	6.9%
		T2	1.28E+05				T2	1.96E+05		
	52.5	T1	1.26E+06	62%		52.5	T1	5.29E+05	99%	
		T2	7.79E+05				T2	5.23E+05		

Supplementary material - Table 2: *Responses (peptide peak area divided by labeled peptide peak area) for soy, milk, egg, and peanut allergens in the eight matrices (chocolate, compote, jam, cookie, spice, mayonnaise, and sauce). The relative standard deviation (RSD) was calculated for the eight matrices or for the matrices belonging to the carbohydrate group (> 50 % carbohydrate: chocolate, compote, jam, cookie, and spices).*

Soy EAFGVNMQIVR Concentration 105 mg soy proteins per kg

	Chocolate	**Compote**	**Jam**	**Cookie**	**Spice**	Ham	Mayonnaise	Sauce	RSD
Response correction IS 1	3.69	3.78	3.86	4.29	3.90	3.47	1.61	1.17	36.0%
	3.69	3.78	3.86	4.29	3.90	-	-	-	5.9%
Response correction IS 2	21.18	6.56	7.74	4.71	15.21	10.67	3.17	1.02	76.1%
	21.18	6.56	7.74	4.71	15.21	-	-	-	62.4%

Milk FFVAPFPEVFGK Concentration 10.5 mg of milk proteins per kg

	Chocolate	**Compote**	**Jam**	**Cookie**	**Spice**	Ham	Mayonnaise	Sauce	RSD
Response correction IS 1	8.42	8.08	7.24	8.22	9.77	7.86	4.90	2.43	32.9%
	8.42	8.08	7.24	8.22	9.77	-	-	-	10.9%
Response correction IS 2	30.17	5.22	8.23	4.84	7.66	4.83	3.13	1.51	111.5%
	30.17	5.22	8.23	4.84	7.66		-	-	95.3%

Egg GGLEPINFQTAADQAR Concentration 15.75 mg egg proteins per kg

	Chocolate	**Compote**	**Jam**	**Cookie**	**Spice**	Ham	Mayonnaise	Sauce	RSD
Response correction IS 1	6.05	7.96	7.06	8.19	11.65	11.75	2.05	-	42.8%
	6.05	7.96	7.06	8.19	11.65	-	-	-	25.9%
Response correction IS 2	12.69	7.38	7.62	8.43	11.45	9.80	2.21	-	40.0%
	12.69	7.38	7.62	8.43	11.45	-	-	-	25.3%

Peanut TANELNLLILR Concentration 52.2mg peanut proteins per kg

	Chocolate	**Compote**	**Jam**	**Cookie**	**Spice**	Ham	Mayonnaise	Sauce	RSD
Response correction IS 1	1.20	1.40	1.71	1.20	1.37	1.55	0.28	0.54	42.6%
	1.20	1.40	1.71	1.20	1.37	-	-	-	15.1%
Response correction IS 2	3.29	2.08	2.35	1.97	6.26	5.49	0.69	0.54	73.6%
	3.29	2.08	2.35	1.97	6.26	-	-	-	56.2%

Supplementary material - Table 3-A: *The slope and intercept of the regression line obtained without internal standard correction or after correction with labelled IS 1 and IS 2 peptides, were used to determine the initial concentration of milk, egg, soy, and peanut allergens at the LOQ: 0.5 mg milk proteins, 0.75 mg egg proteins, 5 mg soy proteins, and 2.5 mg peanut proteins per kg food product. The concentration was calculated by dividing the intercept by the slope. The recovery was obtained by dividing the experimental concentration by the theoretical concentration.*

Without correction IS LOQ

		Chocolate	Compote	Jam	Cookie	Spice	Ham	Mayonnaise	Sauce
Soy EAFGVNMQIVR	Slope	1488	4952	8504	1656	1114	1535	981	830
	Intercept	7614	12419	76978	12869	4344	11834	3350	3650
	Concentration (mg/kg)	5.12	2.51	9.05	7.77	3.90	7.71	3.42	4.40
	Recovery (%)	102.4	**50.2**	**181.0**	**155.4**	78.0	**154.1**	68.3	87.9
	R^2	0.984	0.996	0.995	0.997	0.972	0.994	0.993	0.998
Milk FFVAPFPEVFGK	Slope	24742	117476	160990	34938	19798	127167	92545	44606
	Intercept	12433	68405	161052	16432	11016	94950	34298	18894
	Concentration (mg/kg)	0.50	0.58	1.00	0.47	0.56	0.75	0.37	0.42
	Recovery (%)	100.5	116.5	**200.1**	94.1	111.3	**149.3**	74.1	84.7
	R^2	0.994	1.000	0.992	0.999	0.995	0.986	0.991	0.999
Egg GGLEPINFQTAADQAR	Slope	6606	21634	34990	7486	7768	9468	1524	93000
	Intercept	5456	14016	38126	7728	6705	10091	-93	5509404
	Concentration (mg/kg)	0.83	0.65	1.09	1.03	0.86	1.07	-0.06	59.24
	Recovery (%)	110.1	86.4	**145.3**	**137.6**	115.1	**142.1**	-	-
	R^2	0.991	1.000	0.998	1.000	1.000	0.994	-	-
Peanut TANELNLLILR	Slope	1818	5637	9302	1016	866	2471	715	1132
	Intercept	3794	10178	47225	3697	3216	8410	-202	2287
	Concentration (mg/kg)	2.09	1.81	5.08	3.64	3.71	3.40	-0.28	2.02
	Recovery (%)	83.5	72.2	**203.1**	**145.5**	**148.5**	**136.2**	-	80.8
	R^2	0.998	0.999	0.993	0.997	0.992	0.993	-	0.999

Correction IS 1 **LOQ**

		Chocolate	Compote	Jam	Cookie	Spice	Ham	Mayonnaise	Sauce
Soy EAFGVNMQIVR	Slope	0.035	0.036	0.037	0.041	0.037	0.143	0.015	0.011
	Intercept	0.212	0.144	0.154	0.155	0.277	1.386	0.074	0.059
	Concentration (mg/kg)	6.03	4.00	4.20	3.75	7.41	9.70	4.92	5.23
	Recovery (%)	**120.5**	80.0	84.0	75.0	**148.3**	**194.1**	98.4	104.6
	R^2	0.999	0.999	0.999	0.998	0.990	0.989	0.996	0.999
Milk FFVAPFPEVFGK	Slope	0.801	0.772	0.691	0.785	0.923	0.726	0.458	0.232
	Intercept	0.411	0.391	0.236	0.388	0.616	0.431	0.136	0.122
	Concentration (mg/kg)	0.51	0.51	0.34	0.49	0.67	0.59	0.30	0.53
	Recovery (%)	102.5	101.3	68.4	98.8	**133.5**	118.9	**59.3**	105.7
	R^2	1.000	1.000	0.998	1.000	0.999	1.000	0.987	1.000
Egg GGLEPINFQTAADQAR	Slope	0.373	0.505	0.450	0.522	0.741	0.702	0.132	0.704
	Intercept	0.550	0.391	0.289	0.378	0.535	0.793	0.005	63.514
	Concentration (mg/kg)	1.47	0.77	0.64	0.73	0.72	1.13	0.04	90.23
	Recovery (%)	**196.6**	103.3	85.7	96.7	96.3	**150.6**	-	-
	R^2	0.992	1.000	1.000	1.000	1.000	0.997	-	-
Peanut TANELNLLILR	Slope	0.023	0.027	0.032	0.023	0.026	0.117	0.005	0.010
	Intercept	0.060	0.058	0.076	0.068	0.093	0.461	-0.001	0.029
	Concentration (mg/kg)	2.61	2.19	2.36	2.98	3.64	3.94	-0.22	2.76
	Recovery (%)	104.5	87.5	94.4	119.1	**145.6**	**157.5**	-	110.5
	R^2	1.000	1.000	0.999	0.999	1.000	0.998	-	0.998

Correction IS 2 **LOQ**

		Chocolate	Compote	Jam	Cookie	Spice	Ham	Mayonnaise	Sauce
Soy EAFGVNMQIVR	Slope	0.205	0.062	0.073	0.045	0.032	0.101	0.030	0.010
	Intercept	1.160	0.408	0.495	0.254	0.178	0.629	0.095	0.056
	Concentration (mg/kg)	5.67	6.59	6.81	5.63	5.46	6.25	3.14	5.73
	Recovery (%)	113.4	**131.8**	**136.2**	112.7	109.3	**125.0**	62.7	114.7
	R^2	0.996	1.000	1.000	0.998	0.998	1.000	0.995	0.999
Milk FFVAPFPEVFGK	Slope	2.892	0.498	0.778	0.463	0.744	0.456	0.292	0.144
	Intercept	0.542	0.248	0.410	0.242	0.402	0.245	0.093	0.077
	Concentration (mg/kg)	0.19	0.50	0.53	0.52	0.54	0.54	0.32	0.53
	Recovery (%)	**37.5**	99.9	105.4	104.4	108.1	107.7	63.7	106.9
	R^2	0.996	1.000	0.999	1.000	1.000	1.000	0.988	1.000
Egg GGLEPINFQTAADQAR	Slope	0.802	0.466	0.481	0.539	0.733	0.612	0.141	1.079
	Intercept	0.563	0.360	0.350	0.367	0.580	0.589	-0.051	93.273
	Concentration (mg/kg)	0.70	0.77	0.73	0.68	0.79	0.96	-0.36	86.44
	Recovery (%)	93.6	103.1	96.9	90.6	105.5	**128.3**	-	-
	R^2	0.998	1.000	0.999	0.999	0.998	0.999	-	-
Peanut TANELNLLILR	Slope	0.063	0.039	0.044	0.038	0.029	0.102	0.013	0.010
	Intercept	0.109	0.114	0.116	0.087	0.076	0.312	0.007	0.026
	Concentration (mg/kg)	1.72	2.90	2.62	2.31	2.60	3.04	0.55	2.53
	Recovery (%)	69.0	115.8	104.7	92.6	104.1	**121.7**	-	101.1
	R^2	1.000	1.000	0.999	1.000	0.999	0.998	-	1.000

Supplementary material - Table 3-B: *The slope and intercept of the regression line, obtained without internal standard correction and after correction with labeled IS 1 or IS 2 peptides, were used to determine the initial concentrations of milk, egg, soy, and peanut allergens at 10 x LOQ: 5 mg milk proteins, 7.5 mg egg proteins, 50 mg soy proteins, and 25 mg peanut per kg food product. The concentration was calculated by dividing the intercept by the slope and the recovery was obtained by dividing the experimental concentration by the theoretical concentration.*

Without correction IS **10 x LOQ**

		Chocolate	Compote	Jam	Cookie	Spice	Ham	Mayonnaise	Sauce
Soy EAFGVNMQIVR	Slope	2111	1871	3800	1583	692	0.018	0.034	0.061
	Intercept	117652	108798	382237	59018	40405	0.812	1.515	2.918
	Concentration (mg/kg)	55.73	58.16	100.58	37.28	58.37	45.15	44.03	47.78
	Recovery (%)	111.5	116.3	**201.2**	74.6	116.7	90.3	88.1	95.6
	R^2	0.995	0.998	**0.928**	**0.945**	**0.945**	0.998	0.989	0.996
Milk FFVAPFPEVFGK	Slope	40544	65350	136907	75448	48755	0.491	0.319	0.563
	Intercept	167372	297597	715610	473879	415895	2.679	1.778	2.663
	Concentration (mg/kg)	4.13	4.55	5.23	6.28	8.53	5.45	5.57	4.73
	Recovery (%)	82.6	91.1	104.5	**125.6**	**170.6**	109.1	111.4	94.7
	R^2	0.975	0.991	**0.950**	0.995	**0.913**	0.995	0.999	1.000
Egg GGLEPINFQTAADQAR	Slope	10507	8003	11634	6113	1238	0.247	0.190	-0.712
	Intercept	60273	68362	209849	69037	7496	0.875	0.829	677.878
	Concentration (mg/kg)	5.74	8.54	18.04	11.29	6.05	3.54	4.36	-951.49
	Recovery (%)	76.5	113.9	**240.5**	**150.6**	80.7	**47.1**	**58.2**	-
	R^2	**0.930**	0.990	**0.870**	0.989	0.984	0.983	0.989	-
Peanut TANELNLLILR	Slope	2839	1391	5943	1942	532	0.007	0.018	0.038
	Intercept	74503	39410	197852	48657	12125	0.126	0.346	0.997
	Concentration (mg/kg)	26.24	28.32	33.29	25.05	22.80	18.68	19.64	26.53
	Recovery (%)	105.0	113.3	**133.2**	100.2	91.2	74.7	78.6	106.1
	R^2	**0.940**	0.993	0.984	**0.969**	**0.959**	0.995	0.989	0.998

Correction IS 1 **10 x LOQ**

		Chocolate	Compote	Jam	Cookie	Spice	Ham	Mayonnaise	Sauce
Soy EAFGVNMQIVR	Slope	0.020	0.011	0.035	0.021	0.022	0.036	0.018	0.046
	Intercept	1.046	0.590	1.903	1.050	1.172	1.687	0.812	2.120
	Concentration (mg/kg)	53.61	54.33	54.15	49.47	53.85	46.33	45.15	46.42
	Recovery (%)	107.2	108.7	108.3	98.9	107.7	92.7	90.3	92.8
	R^2	0.995	0.997	0.998	0.994	0.989	0.997	0.998	0.997
Milk FFVAPFPEVFGK	Slope	0.489	0.189	0.668	0.563	0.615	0.793	0.491	0.840
	Intercept	2.406	0.927	3.320	2.617	3.085	3.969	2.679	4.268
	Concentration (mg/kg)	4.92	4.90	4.97	4.65	5.02	5.00	5.45	5.08
	Recovery (%)	98.4	97.9	99.3	92.9	100.4	100.1	109.1	101.6
	R^2	0.997	1.000	0.999	1.000	0.999	0.999	0.995	0.998
Egg GGLEPINFQTAADQAR	Slope	0.106	0.072	0.406	0.110	0.142	0.791	0.247	1.131
	Intercept	0.715	0.528	3.703	0.793	1.050	6.993	0.875	253.002
	Concentration (mg/kg)	6.76	7.35	9.12	7.19	7.39	8.84	3.54	223.72
	Recovery (%)	90.1	98.0	**121.6**	95.9	98.6	117.9	**47.1**	-
	R^2	**0.921**	0.997	0.992	0.998	0.978	0.984	0.983	-
Peanut TANELNLLILR	Slope	0.017	0.010	0.029	0.016	0.020	0.025	0.007	0.026
	Intercept	0.471	0.254	0.765	0.435	0.462	0.699	0.126	0.680
	Concentration (mg/kg)	27.91	25.41	25.95	26.99	23.06	27.86	18.68	26.20
	Recovery (%)	111.6	101.6	103.8	108.0	92.2	111.5	74.7	104.8
	R^2	0.998	0.999	0.999	1.000	0.996	0.998	0.995	0.999

Correction IS 2 **10 x LOQ**

		Chocolate	Compote	Jam	Cookie	Spice	Ham	Mayonnaise	Sauce
Soy EAFGVNMQIVR	Slope	0.032	0.011	0.087	0.019	0.040	0.132	0.034	0.061
	Intercept	1.612	0.603	4.102	0.813	1.512	5.806	1.515	2.918
	Concentration (mg/kg)	49.73	55.79	47.24	42.28	37.69	44.11	44.03	47.78
	Recovery (%)	99.5	111.6	94.5	84.6	75.4	88.2	88.1	95.6
	R^2	**0.969**	0.998	0.998	0.978	**0.951**	0.987	0.989	0.996
Milk FFVAPFPEVFGK	Slope	0.964	0.124	0.778	0.316	0.426	0.518	0.319	0.563
	Intercept	2.808	0.610	4.704	1.519	2.251	2.568	1.778	2.663
	Concentration (mg/kg)	2.91	4.92	6.04	4.81	5.28	4.96	5.57	4.73
	Recovery (%)	**58.2**	98.3	**120.9**	96.2	105.7	99.2	111.4	94.7
	R^2	0.988	1.000	0.975	1.000	0.998	0.999	0.999	1.000
Egg GGLEPINFQTAADQAR	Slope	0.328	0.093	0.454	0.149	0.245	0.851	0.190	-0.712
	Intercept	1.167	0.659	4.076	1.078	1.726	6.123	0.829	677.878
	Concentration (mg/kg)	3.56	7.06	8.98	7.25	7.05	7.19	4.36	-951.49
	Recovery (%)	**47.5**	94.2	119.8	96.6	94.0	95.9	**58.2**	-
	R^2	**0.915**	1.000	0.994	0.998	0.988	0.997	0.989	-
Peanut TANELNLLILR	Slope	0.021	0.009	0.049	0.015	0.062	0.099	0.018	0.038
	Intercept	0.456	0.253	1.142	0.414	1.569	2.895	0.346	0.997
	Concentration (mg/kg)	21.27	26.87	23.40	27.55	25.50	29.20	19.64	26.53
	Recovery (%)	85.1	107.5	93.6	110.2	102.0	116.8	78.6	106.1
	R^2	0.998	0.999	0.998	0.999	0.989	0.985	0.989	0.998

Supplementary material - Table 4-A: *The slope and intercept of the regression line, obtained without internal standard correction and after correction with labeled IS 1 or IS 2 peptides in three replicates of compote, were used to determine the initial concentration of milk, egg, soy, and peanut allergens at 10 x LOQ: per kg of food product, 5 mg for milk proteins, 7.5 mg for egg proteins, 50 mg for soy proteins, and 25 mg for peanut. The concentration was calculated by dividing the intercept by the slope. The recovery was obtained by dividing the experimental concentration by the theoretical concentration.*

		Compote R1			Compote R2			Compote R3		
		Without correction	IS correction		Without correction	IS correction		Without correction	IS correction	
			IS 1	IS 2		IS 1	IS 2		IS 1	IS 2
Soy	Slope	1316	0.011	0.011	1120	0.010	0.011	1459	0.012	0.011
	Intercept	76399	0.590	0.603	96859	0.579	0.597	71709	0.584	0.611
	Concentration (mg/kg)	58.06	54.33	55.79	86.51	56.22	55.81	49.13	50.42	54.36
	Recovery (%)	116.1	108.7	111.6	**173.0**	112.4	111.6	98.3	100.8	108.7
	R^2	0.999	0.997	0.998	**0.955**	0.997	0.998	0.995	0.999	0.999
Milk	Slope	44876	0.189	0.124	42384	0.182	0.118	48034	0.184	0.122
	Intercept	247089	0.927	0.610	291732	0.938	0.613	240618	1.009	0.653
	Concentration (mg/kg)	5.51	4.90	4.92	6.88	5.14	5.18	5.01	5.48	5.36
	Recovery (%)	110.1	97.9	98.3	**137.7**	102.8	103.6	100.2	109.7	107.2
	R^2	0.996	1.000	1.000	**0.956**	0.997	0.998	0.992	0.982	0.985
Egg	Slope	9210	0.072	0.093	8145	0.067	0.080	10646	0.072	0.089
	Intercept	80853	0.528	0.659	102196	0.539	0.691	74897	0.556	0.704
	Concentration (mg/kg)	8.78	7.35	7.06	12.55	8.08	8.60	7.04	7.66	7.92
	Recovery (%)	117.1	98.0	94.2	**167.3**	107.8	114.7	93.8	102.2	105.5
	R^2	0.995	0.997	1.000	0.982	0.998	0.995	0.999	0.999	0.996
Peanut	Slope	1758	0.010	0.009	1636	0.010	0.009	2056	0.010	0.009
	Intercept	49212	0.254	0.253	57801	0.252	0.251	46816	0.256	0.256
	Concentration (mg/kg)	27.99	25.41	26.87	35.33	24.89	26.52	22.77	24.84	27.07
	Recovery (%)	111.9	101.6	107.5	**141.3**	99.5	106.1	91.1	99.4	108.3
	R^2	0.997	0.999	0.999	**0.941**	0.998	0.999	0.998	0.999	0.998

Supplementary material - Table 4-B: Mean of the concentration obtained for the three replicates of compote for milk, egg, soy and peanut proteins without internal standard correction and with labeled IS 1 or IS 2 peptide correction. The RSD between the three replicates and the mean recovery were also determined.

	Mean concentration compote			**Mean recovery compote**			**RSD (%)**		
	Without correction	**IS correction**		**Without correction**	**IS correction**		**Without correction**	**IS correction**	
		IS 1	IS 2		IS 1	IS 2		IS 1	IS 2
Soy	64.6	53.7	55.3	**129%**	107%	111%	25%	4%	1%
Milk	5.8	5.2	5.2	116%	103%	103%	14%	5%	4%
Egg	9.5	7.7	7.9	**126%**	103%	105%	24%	4%	8%
Peanut	28.7	25.0	26.8	115%	100%	107%	18%	1%	1%

Supplementary material - Table 5-A: *The slope and intercept of the regression line obtained with 3 points of calibration, without internal standard correction and after correction with labeled IS 1 and IS 2 peptides, were used to determine the initial concentration of milk, egg, soy, and peanut allergens at the LOQ: 0.5 mg milk proteins, 0.75 mg egg proteins, 5 mg soy proteins, and 2.5 mg peanut proteins per kg food product. The concentration was calculated by dividing the intercept by the slope and the recovery was obtained by dividing the experimental concentration by the theoretical concentration*

Without correction IS **LOQ**

		Chocolate	Compote	Jam	Cookie	Spice	Ham	Mayonnaise	Sauce
Soy EAFGVNMQIVR	Slope	1760	4396	9561	1821	1383	1348	880	833
	Intercept	7520	18607	56756	11058	2824	12556	6127	4271
	Concentration (mg/kg)	4.3	4.2	5.9	6.1	2.0	9.3	7.0	5.1
	Recovery (%)	85.5	84.7	118.7	**121.5**	**40.8**	**186.3**	**139.2**	102.6
	R^2	0.999	1.000	1.000	0.999	0.998	0.997	0.999	0.998
Milk FFVAPFPEVFGK	Slope	26377	120801	186000	33296	21266	104085	76165	44421
	Intercept	15044	64771	116535	16479	7849	101953	53250	22507
	Concentration (mg/kg)	0.6	0.5	0.6	0.5	0.4	1.0	0.7	0.5
	Recovery (%)	114.1	107.2	**125.3**	99.0	73.8	**195.9**	**139.8**	101.3
	R^2	0.999	1.000	1.000	1.000	1.000	0.997	1.000	1.000
Egg GGLEPINFQTAADQAR	Slope	5563	21486	33288	7520	7779	8308	1245	-26712
	Intercept	6342	15767	37418	7691	6361	11062	536	5843930
	Concentration (mg/kg)	1.1	0.7	1.1	1.0	0.8	1.3	-	-
	Recovery (%)	**152.0**	97.8	**149.9**	**136.4**	109.0	**177.5**	-	-
	R^2	0.998	1.000	1.000	1.000	1.000	0.997	-	-
Peanut TANELNLLILR	Slope	1740	5228	10635	1077	960	2110	498	1157
	Intercept	5019	12842	34885	3597	3034	10259	1757	2433
	Concentration (mg/kg)	2.9	2.5	3.3	3.3	3.2	4.9	-	2.1
	Recovery (%)	115.4	98.3	**131.2**	**133.7**	**126.4**	**194.5**	-	84.1
	R^2	1.000	1.000	1.000	0.995	1.000	0.997	-	1.000

Correction IS 1 **LOQ**

		Chocolate	Compote	Jam	Cookie	Spice	Ham	Mayonnaise	Sauce
Soy EAFGVNMQIVR	Slope	0.04	0.03	0.03	0.04	0.04	0.03	0.01	0.01
	Intercept	0.19	0.16	0.17	0.19	0.21	0.20	0.10	0.06
	Concentration (mg/kg)	5.1	4.6	4.8	4.6	4.7	6.5	7.4	5.1
	Recovery (%)	102.9	91.4	96.7	91.1	93.9	**130.3**	**148.5**	102.3
	R^2	1.000	1.000	1.000	1.000	1.000	0.999	0.996	0.998
Milk FFVAPFPEVFGK	Slope	0.80	0.78	0.65	0.80	0.97	0.73	0.36	0.24
	Intercept	0.43	0.38	0.30	0.36	0.56	0.41	0.23	0.12
	Concentration (mg/kg)	0.5	0.5	0.5	0.5	0.6	0.6	0.6	0.5
	Recovery (%)	106.4	97.2	93.2	90.8	116.0	112.6	**127.4**	100.9
	R^2	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
Egg GGLEPINFQTAADQAR	Slope	0.40	0.51	0.45	0.53	0.73	0.67	0.11	-0.51
	Intercept	0.41	0.37	0.30	0.36	0.56	0.63	0.05	64.68
	Concentration (mg/kg)	1.0	0.7	0.7	0.7	0.8	0.9	-	-
	Recovery (%)	**137.3**	97.8	89.0	90.4	101.1	**124.5**	-	-
	R^2	0.998	1.000	1.000	0.999	1.000	1.000	-	-
Peanut TANELNLLILR	Slope	0.02	0.03	0.03	0.02	0.03	0.03	0.00	0.01
	Intercept	0.06	0.06	0.08	0.06	0.09	0.09	0.01	0.02
	Concentration (mg/kg)	2.6	2.5	2.6	2.4	3.3	3.3	-	2.0
	Recovery (%)	102.2	99.8	104.4	97.0	**133.6**	**131.1**	-	81.1
	R^2	1.000	1.000	1.000	1.000	1.000	1.000	-	1.000

Correction IS 2 **LOQ**

		Chocolate	Compote	Jam	Cookie	Spice	Ham	Mayonnaise	Sauce
Soy EAFGVNMQIVR	Slope	0.23	0.06	0.07	0.05	0.16	0.10	0.03	0.01
	Intercept	0.83	0.35	0.46	0.20	0.99	0.59	0.18	0.05
	Concentration (mg/kg)	3.6	5.6	6.1	4.4	6.1	5.8	6.5	5.6
	Recovery (%)	72.8	111.0	**122.8**	87.2	**121.4**	115.9	**130.8**	111.2
	R^2	0.999	1.000	1.000	1.000	1.000	1.000	0.999	0.999
Milk FFVAPFPEVFGK	Slope	2.53	0.50	0.76	0.48	0.74	0.44	0.23	0.15
	Intercept	1.12	0.25	0.45	0.22	0.40	0.25	0.15	0.07
	Concentration (mg/kg)	0.4	0.5	0.6	0.5	0.5	0.6	0.6	0.5
	Recovery (%)	88.9	100.5	118.5	92.4	109.3	115.4	**127.4**	100.7
	R^2	1.000	1.000	0.998	1.000	1.000	1.000	1.000	1.000
Egg GGLEPINFQTAADQAR	Slope	0.76	0.46	0.47	0.54	0.66	0.59	0.09	-1.25
	Intercept	0.59	0.35	0.36	0.35	0.74	0.61	0.05	96.56
	Concentration (mg/kg)	0.8	0.8	0.8	0.6	1.1	1.0	-	-
	Recovery (%)	103.3	102.2	102.1	85.7	**150.8**	**137.5**	-	-
	R^2	0.999	1.000	1.000	0.999	0.999	0.999	-	-
Peanut TANELNLLILR	Slope	0.06	0.04	0.04	0.04	0.12	0.10	0.01	0.01
	Intercept	0.13	0.11	0.12	0.09	0.41	0.34	0.03	0.02
	Concentration (mg/kg)	2.1	2.9	2.9	2.4	3.3	3.6	-	2.2
	Recovery (%)	82.7	114.3	117.6	95.8	**133.0**	**143.2**	-	89.5
	R^2	1.000	1.000	1.000	1.000	1.000	1.000	-	1.000

Supplementary material - Table 5-B: *The slope and intercept of the regression line obtained with 3 points of calibration, without internal standard correction and after correction with labeled IS 1 or IS 2 peptides, were used to determine the initial concentration of milk, egg, soy, and peanut allergens at 10 x LOQ: 5 mg milk proteins, 7.5 mg egg proteins, 50 mg soy proteins, and 25 mg peanut proteins per kg food product. The concentration was calculated by dividing the intercept by the slope and the recovery was obtained by dividing the experimental concentration by the theoretical concentration.*

Without correction IS **10 x LOQ**

		Chocolate	Compote	Jam	Cookie	Spice	Ham	Mayonnaise	Sauce
Soy EAFGVNMQIVR	Slope	2053	1361	3658	1887	969	920	820	1063
	Intercept	121323	75246	388029	107849	47501	68097	43065	64728
	Concentration (mg/kg)	59.1	55.3	106.1	57.2	49.0	74.1	52.5	60.9
	Recovery (%)	118.2	110.6	**212.2**	114.3	98.1	**148.1**	105.0	**121.8**
	R^2	0.985	0.997	0.989	1.000	0.997	0.988	0.984	0.950
Milk FFVAPFPEVFGK	Slope	49522	40844	128942	67463	22125	71760	67904	70818
	Intercept	143409	250804	814592	278376	96924	495141	417123	437583
	Concentration (mg/kg)	2.9	6.1	6.3	4.1	4.4	6.9	6.1	6.2
	Recovery (%)	**57.9**	**122.8**	**126.3**	82.5	87.6	**138.0**	**122.9**	**123.6**
	R^2	0.984	0.996	0.991	0.999	1.000	0.999	0.961	0.989
Egg GGLEPINFQTAADQAR	Slope	6961	7985	10496	7448	7243	5730	933	-109811
	Intercept	57835	82808	229380	66847	43013	72515	8176	14407731
	Concentration (mg/kg)	8.3	10.4	21.9	9.0	5.9	12.7	8.8	-
	Recovery (%)	110.8	**138.3**	**291.4**	119.7	79.2	**168.7**	116.8	-
	R^2	0.996	0.999	0.981	0.994	0.999	0.990	0.999	-
Peanut TANELNLLILR	Slope	3994	1844	5748	1409	614	1388	631	1633
	Intercept	63115	48327	208731	37796	12682	55678	12844	35417
	Concentration (mg/kg)	15.8	26.2	36.3	26.8	20.6	40.1	20.4	21.7
	Recovery (%)	63.2	104.8	**145.3**	107.3	82.6	**160.4**	81.5	86.7
	R^2	0.990	0.993	0.999	0.995	0.995	0.999	0.989	0.998

Correction IS 1 **10 x LOQ**

		Chocolate	Compote	Jam	Cookie	Spice	Ham	Mayonnaise	Sauce
Soy EAFGVNMQIVR	Slope	0.02	0.01	0.04	0.02	0.03	0.04	0.02	0.05
	Intercept	1.06	0.57	1.86	1.09	1.12	1.64	0.80	2.03
	Concentration (mg/kg)	59.4	50.9	52.5	57.0	43.2	45.5	41.6	42.9
	Recovery (%)	118.8	101.9	105.1	114.0	86.5	91.0	83.2	85.7
	R^2	0.990	0.991	1.000	0.994	0.996	1.000	0.997	0.999
Milk FFVAPFPEVFGK	Slope	0.51	0.19	0.67	0.56	0.63	0.85	0.53	0.89
	Intercept	2.35	0.93	3.35	2.62	3.10	3.90	2.61	4.23
	Concentration (mg/kg)	4.6	4.9	5.0	4.7	4.9	4.6	4.9	4.8
	Recovery (%)	92.4	97.4	100.4	93.2	98.8	91.8	98.1	95.5
	R^2	0.997	1.000	1.000	1.000	0.997	1.000	1.000	1.000
Egg GGLEPINFQTAADQAR	Slope	0.09	0.08	0.40	0.12	0.17	0.97	0.19	1.96
	Intercept	0.64	0.52	3.84	0.79	1.03	6.74	0.99	249.83
	Concentration (mg/kg)	7.5	6.8	9.6	6.6	6.0	6.9	5.2	-
	Recovery (%)	99.6	90.2	**128.3**	87.8	79.8	92.5	68.8	-
	R^2	1.000	1.000	0.995	1.000	0.992	0.999	0.990	-
Peanut TANELNLLILR	Slope	0.02	0.01	0.03	0.02	0.02	0.03	0.01	0.02
	Intercept	0.47	0.25	0.77	0.43	0.47	0.70	0.13	0.69
	Concentration (mg/kg)	28.0	23.7	26.3	27.5	24.8	27.4	22.6	28.2
	Recovery (%)	112.0	94.7	105.3	109.9	99.4	109.5	90.2	113.0
	R^2	0.999	1.000	0.999	1.000	0.995	0.999	1.000	1.000

Correction IS 2 **10 x LOQ**

		Chocolate	Compote	Jam	Cookie	Spice	Ham	Mayonnaise	Sauce
Soy EAFGVNMQIVR	Slope	0.03	0.01	0.09	0.02	0.03	0.14	0.04	0.06
	Intercept	1.81	0.59	4.10	0.81	1.61	5.81	1.53	2.87
	Concentration (mg/kg)	60.4	51.0	47.3	35.0	61.7	41.8	39.9	48.7
	Recovery (%)	**120.8**	102.0	94.5	69.9	**123.3**	83.6	79.8	97.5
	R^2	0.996	0.999	0.998	1.000	0.990	0.998	0.998	0.995
Milk FFVAPFPEVFGK	Slope	0.81	0.13	0.81	0.31	0.40	0.55	0.33	0.58
	Intercept	2.82	0.61	4.35	1.52	2.31	2.52	1.76	2.65
	Concentration (mg/kg)	3.5	4.8	5.4	4.9	5.8	4.5	5.3	4.6
	Recovery (%)	69.2	96.7	107.3	97.6	115.5	91.0	105.7	91.7
	R^2	0.997	1.000	0.999	1.000	0.997	1.000	0.999	1.000
Egg GGLEPINFQTAADQAR	Slope	0.20	0.09	0.45	0.14	0.25	0.84	0.16	1.85
	Intercept	1.10	0.66	4.16	1.08	1.84	6.24	0.92	681.08
	Concentration (mg/kg)	5.5	7.2	9.3	7.7	7.4	7.4	5.9	-
	Recovery (%)	72.8	96.3	**123.7**	103.3	99.1	99.1	79.0	-
	R^2	0.996	1.000	0.996	1.000	0.997	0.998	0.988	-
Peanut TANELNLLILR	Slope	0.02	0.01	0.05	0.01	0.06	0.12	0.02	0.04
	Intercept	0.44	0.25	1.16	0.42	1.57	2.87	0.36	0.99
	Concentration (mg/kg)	20.3	25.4	23.8	29.1	27.8	24.5	23.1	27.9
	Recovery (%)	81.1	101.7	95.1	116.6	111.1	98.1	92.3	111.4
	R^2	1.000	1.000	1.000	0.999	0.977	0.999	0.985	0.998

CONCLUSIN AND PROPECTS

I Detection of allergen marker peptides by mass spectrometry

This project, carried out in a routine laboratory, aimed to provide a tool for confirming results obtained with ELISAs for the detection of allergens. Indeed, allergen detection by ELISA in processed food products is often compromised because of protein denaturation, causing a substantial loss of method sensitivity (Dumont et al., 2010; Albillos, 2012). Moreover, interfering compounds due to the complexity of food matrices even sometimes prevent allergen protein detection (Poms et al., 2004). Consequently, any confirmatory method used must be highly sensitive and specific in order to detect allergens in processed and complex food products. To avoid the various problems encountered with ELISAs, mass spectrometry was chosen as a routine alternative and/or confirmatory technique.

The main objective of this work was to develop a single method based on ultra-high performance liquid chromatography coupled to tandem mass spectrometry UHPLC-MS/MS, for the detection of peptides from egg (white and yolk), milk (whey and casein), soy, peanut, almond, hazelnut, walnut, cashew, pecan nut and pistachio allergens after an enzymatic digestion of proteins in specific food products. The target food matrix in this project was cookie because it is a complex matrix and subjected to high-temperature processing, but three other food matrices were also later added/tested: tomato sauce (acidic - 95°C – 45 min), banana ice cream (fatty), and chocolate (rich in tannins). The additional matrices expand the range of food products–processed or not–that can be analyzed, providing considerable added value for routine laboratories. The target allergens in this project were milk (casein and whey), egg (white and yolk), soy, and peanut allergens. As a second step, the method was implemented with six tree nut allergens (almond, hazelnut, walnut, pecan nut, cashew, and pistachio).

I.I Selection of marker peptides

The first step in developing the method was to select marker peptides originating from enzymatic digestion of proteins. Two possibilities were considered (a) identifying marker peptides by high-resolution mass spectrometry (HRMS) (Faeste et al., 2009; Monaci et al., 2009) or (b) an *in silico* approach with UHPLC-MS/MS (Ansari et al., 2011; Korte et al., 2016).

The *in silico* approach consists in retrieving target protein sequences from a database (e.g. Uniprot), performing an *in silico* digestion of proteins (e. g. with Skyline), and predicting MS/MS parameters. A list of criteria set by the user, such as peptide length (8 to 25 amino acids), digestive enzyme (trypsin), charge states of the peptide (+2, +3), and fragmentation (b, y) allow generating a theoretical list of peptides and several hundred of MRM transitions per protein (MacLean et al., 2010). Once such a list was obtained, sample extracts were analyzed with a triple quadrupole mass spectrometer to experimentally select MRM transitions. Proteins were extracted from raw ingredients and then digested with trypsin, which is a serine protease that specifically cleaves after lysine (K) and arginine (R) residues (Olsen et al., 2004). Peptide extracts were analyzed by UHPLC-MS/MS in order to select

only the most abundant peptides and 3 or 4 transitions per peptide. Then cookies containing 1350 mg of milk proteins, 3250 mg of egg proteins, 2100 mg of soy proteins, and 1250 mg of peanut proteins per kg were analyzed and a list of peptides robust to the thermal process was established. At this step, the collision energy (an MS parameter expressed in volts, adjusted to allow peptide fragmentation in the second quadrupole) was optimized, as were the extraction and purification methods. A final list of peptides was then established by analyzing the three additional incurred matrices (sauce, chocolate, and ice cream). Three to five peptides per allergen with three MRM transitions per peptide were conserved for allergen detection by UHPLC-MS/MS.

To conclude, final marker peptides were selected based on three criteria: the area and intensity of the peak corresponding to target peptides in complex and processed food products, the specificity of MRM transitions in allergen-free products and the specificity of peptide sequences to avoid false positive. The selection of marker peptides mainly depends on the abundance of the protein, the target matrix and the applied process, the protocol of sample preparation, especially the purification steps (e.g. selection of more hydrophobic or more hydrophilic peptides), and the criteria fixed by the user for the selection of marker peptides (e.g. peptide length, no missed cleavages by the enzyme). The abundance of some proteins, such as α-S1 casein (32% of milk proteins), β-lactoglobulin (10% of milk proteins) or ovalbumin (54% of egg proteins) for the analysis of casein, whey milk and egg white, respectively, make easier the selection of marker peptides. Indeed, in almost all other published methods the same marker peptides are chosen: milk α-S1 casein (FFVAPFPEVFGK, YLGYLEQLLR and HQGLPQEVLNELLLR), β-lactoglobulin (LSFNPTQLEEQCHI and VLVLDTDYK) and ovalbumin (GGLEPINFQTAADQAR and LTEWTSSNVMEER) (Heick et al., 2011; Monaci et al., 2014 a; Planque et al., 2016; Pilolli et al., 2017; Boo et al., 2018; New et al., 2018). The high number of both protein/ isoforms and unspecific peptides (unspecific amino acid sequence) for soy, peanut and tree nuts make the selection of peptides more complicated. For those allergens, selection of marker peptides depends on the target matrix and on the sample preparation protocol but no trend list of marker peptides can be clearly established from literature. Indeed, with the same method, the best marker peptides for peanut in chocolate will be different than those in cookies. Consequently, the method able to detect peptides in several matrices will be less sensitive than those developed for a single food matrix.

In this study, peptide modifications such as oxidation, or phosphorylation were not taken into account because modifications are specific to a food product and/or a process while the goal of this study was to develop a method allowing to simultaneously detect peptides of several food allergens in several food matrix.

However, during the selection of marker peptides, we observed a decrease in the detection sensitivity for the analysis of peptides in cookies, especially for egg yolk peptides. The identification of peptide modifications in cookies could improve the sensitivity of the method. Indeed, if the process induces an oxidation of methionine, only peptides without this modification will be detected by UHPLC-MS/MS.

Peptide modifications are currently investigated by high resolution mass spectrometry in another project (ALLERSENS) in the laboratory (Thesis of M. Gavage). A list of modifications depending on the food process will be established and the percentages of unmodified and modified peptides will be determined. When the percentage of modified peptides is high for a specific process applied, this

modification could be systematically included in the protocol (e.g. oxidation of methionine with hydrogen peroxide (Boonvisut, 1982)). In this example, the oxidation yield on methionine amino acid in different matrices has to be controlled such as the impact of the oxidation on the other amino acids and peptide detection.

I.II Optimization of the method

At the beginning of the project, no legal thresholds had been set in Europe or in Belgium. The Voluntary Incidental Trace Allergen Labeling (VITAL) system developed in Australia and New Zealand sets thresholds based on clinical studies determining no and low observable adverse effect levels (NOAELs LOAELs) for allergens. ED_{01} and ED_{05} eliciting doses have been determined by VITAL, allowing protection of 99% and 95% of the allergic population, respectively. On the basis of these references, the target analytical sensitivity thresholds (expressed per kg) are 0.75 mg for egg proteins, 2.5 mg for milk or tree nut proteins, 5 mg for peanut proteins, 25 mg for soybean proteins, and 50 mg for cashew proteins (portion size: 40 g) (Allen et al., 2014 a; Taylor et al., 2014 b). To reach this sensitivity for milk, egg, soy, tree nut and peanut allergens, several extraction parameters were optimized, using processed cookies containing 1350 mg of milk proteins, 3250 mg of egg proteins, 2100 mg of soy proteins, and 1250 mg of peanut proteins per kg. Two criteria were considered: the extraction yield of proteins, determined by BCA, and the peptide peak areas determined by UHPLC-MS/MS. The optimization of extraction and purification steps is crucial to reach this sensitivity.

Four extraction buffers commonly used were tested (Pedreschi et al., 2012; Gomaa et al., 2015; Parker et al., 2015; Pilolli et al., 2017): (1) 200 mM TRIS-HCl, pH 8.2; (2) 50 mM TRIS, 500 mM NaCl, pH 8.2; (3) 50 mM PBS, pH 7.4, and (4) 50 mM NH_4HCO_3, pH 8.2. The addition of detergents (SDS, Triton X-100 and CHAPS) or chaotropic agents (urea) and sample defatting (with acetone) were tested in order to improve the sensitivity of the method (Watanabe et al., 2005; Fu et al., 2010). Most detergents are not compatible with mass spectrometers and should be removed before analysis (Yeung et al., 2008). It turned out that the improvement of the extraction yield afforded by detergents was not enough to compensate for the loss of peptides caused by detergent removal. Chaotropic agents, on the other hand, can denature digestive enzymes and should be diluted to allow optimal protein digestion (Viswanathat et al., 1955; Gabel, 1973). Adding urea significantly improved protein extraction yield, and a compromise was made between maximizing protein extraction yield and avoiding extract dilution. Defatting the cookies with acetone prior to extraction did not improve the sensitivity of the method and added an unnecessary step to the protocol. On the basis of extraction yields and peptide peak areas, we chose the extraction buffer 200 mM TRIS-HCl, pH 8.2 with addition of 2 M urea. The same criteria were used to optimize additional parameters, such as the pH, the extraction time, and the ratio of the amount of sample to amount of sample to the volume of extraction buffer.

The next crucial step after optimization of the extraction was peptide extract purification. Two purification methods were considered: a specific method (immunoaffinity chromatography) and a nonspecific method (solid phase extraction, SPE). After selection of abundant peptides robust to the thermal process, we tested whether they were exposed in the native protein. The synthetic peptides (4 – 5 peptides per allergen) were synthesized by Eurogentec and coupled to a carrier protein (KLH)

for the immunization of rabbits. Normally, rabbits are immunized against whole proteins, but here we wanted to obtain antibodies capable of recognizing the target peptide in the entire protein in both unprocessed and processed food products. All the antibodies were able to recognize the target peptides and at least one antibody per allergen (except for egg) was able to recognize the native protein. The lack of antibodies for the recognition of all four allergens and the promising results obtained in parallel with solid phase extraction purification led us to abandon the immunoaffinity purification strategy. This strategy deserves further investigation, however, as recognition of the native protein in the case of three of the allergens provides encouraging 'proof of concept' of immunization against peptides. In parallel, three SPE cartridges were tested: Sep-pack tC18, Sep-pack C18, and Oasis HLB. The C18 cartridges turned out to allow the best method sensitivity. Several parameters were optimized afterwards, such as the washing solvent for SPE, the elution of peptides from the SPE column, and the temperature of evaporation.

Finally, the enzymatic digestion step was optimized, notably in terms of its duration and the reduction buffer used (TCEP and DTT). The developed method should be both sensitive and applicable in routine laboratories. Optimization of the digestion led to decreasing significantly the time of analysis, permitting the analysis of allergens within a day.

The selection of a single protocol for the analysis of several allergens in a higher number of food products was a real challenge. The most difficult part was the treatment of the high number of data generated by the analysis of more than one hundred of peptides. Indeed, the final list of peptides was determined after the optimization and the analysis of marker peptides in tomato sauce, ice cream and chocolate to ensure the better method sensitivity with the optimized parameters.

The choice we made to use a single protocol for the analysis of peptides belonging to proteins with totally different properties led us to accept compromises and we are aware that the fixed parameters are probably not necessarily the best parameters for each individual allergen. Moreover, the best parameters for the detection of milk peptides determined for cookies will probably be different than those determined for chocolate. However, the use of an optimized protocol specifically depending on the allergen and the matrix to analyze would be extremely costly and unrealistic for routine food laboratory.

The development of the method was done step by step, a change in the order of tested parameters could have also influenced the results as well as the target matrix for the selection of the best parameters. The development of the method was the most challenging part of this project, a lot of compromises have been done and a lot of parameters would still need to be tested that could improve the sensitivity of the method (e.g. size-exclusion chromatography, immunoaffinity column). However, even if the modification of the purification method could lead to a new selection of marker peptides, a method that satisfied to required criteria could be developed and validated.

I.III Sensitivity of the developed method

We have mentioned that, worldwide, food allergy is and remains a global health problem. In order to protect allergic population, reliable analytical methods must be developed. The sensitivity of ELISA

methods for the detection of processed proteins in food products could dramatically be reduced and alternative/complementary methods must thus be developed. However, right now, the question to determine whether UHPLC-MS/MS does guarantee allergen free-products in all kind of food products needs to be addressed.

After optimization of the method, the limits of quantitation reached in two processed matrices (cookie and sauce) and two unprocessed matrices (ice cream and chocolate), expressed in total proteins per kilogram of food, were: 0.5 mg/kg for milk (detection of caseins), 5 mg/kg for milk (detection of whey), 2.5 mg/kg for peanut, hazelnut, pistachio, and cashew proteins, 5 mg/kg for soy, almond, walnut, and pecan nut proteins, 3 mg/kg for egg (detection of egg white), and 60 mg/kg for egg (detection of egg yolk))). The sensitivity reached by our method with a signal-to-noise ratio higher than 10 was below or equal to the VITAL threshold in the case of milk, soy, peanut, hazelnuts, pistachio, and cashew allergens. It exceeded the VITAL threshold in the case of egg, almond, walnut, and pecan allergens. All the target allergens except egg yolk allergens, however, were detected at their respective VITAL thresholds with a signal-to-noise ratio of 3. In **Table 8**, the sensitivity reached by the method is compared with those of the most sensitive state-of-the-art multi-allergen methods. As shown **Table 8**, a real step forward has been done for the detection of food allergen peptides in processed matrices. Indeed, several multi-allergens methods have been developed in incurred and processed food products (Pilolli et al., 2017; Boo et al., 2018; Gu et al., 2018)**;** while few years ago only Heick and collaborators developed a multi-allergen method in processed food products (Heick et al., 2011). Comparing UHPLC-MS/MS methods remains difficult for lack of reference standards and because of differences in reporting units (soluble proteins, proteins, ingredients) and in how method sensitivity is determined (LOD or LOQ). It should be noted, however, that the sensitivity of UHPLC-MS/MS methods has increased significantly, so that mass spectrometry is becoming an instrument of choice for the detection of allergens.

Authors		VITAL	Planque et al. (2017)		Heick et al. (2010)	Pilolli et al. (2017)	Boo et al. (2018)	New et al. (2018)	Gu et al. (2018)
Allergens (% of proteins)	Milk (25.64 - 36%)	**2.5**	0.5 (casein) 5 (whey)	2 (casein) 19.5 (whey)	5	20	5	10	0.2
	Egg (48%)	**0.75**	3 (white) 60 (yolk)	6.3 (white) 125 (yolk)	42	30	5	10	-
	Soy (36 - 53.4%)	**25**	5	9.4	24	19	5	10	1
	Peanut (22 -25 %)	**5**	2.5	11.3	11	40	-	10	2.5
	Almond (20%)	**2.5**	5	25	3	20	-	10	1.3
	Hazelnut (15%)	**2.5**	2.5	16.7	5	-	-	10	1.7
	Walnut (14%)	**50**	5	35.7	70	-	-	10	2
	Cashew (17%)	**2.5**	2.5	15	-	-	-	10	2.3
	Pecan nut (10%)	**2.5**	5	50	-	-	-	10	-
	Pistachio (20%)	**2.5**	2.5	12.5	-	-	-	10	1.3
	Macadamia (8%)	**2.5**	-	-	-	-	-	10	-
	Pine nut (13%)	**2.5**	-	-	-	-	-	10	1.3
	Expression of results	mg of **proteins** per kg	**LOQ** (S/N>10)		**LOD** (S/N>3)	**LOQ** (S/N>10)	**LOD** (S/N>3)	**LOD** (S/N>3)	**LOQ** (S/N>10)
			mg of **proteins** per kg	mg of **ingredients** per kg	mg of **soluble proteins** per kg	mg of **ingredients** per kg	mg of **ingredients** per kg	mg of **ingredients** per kg	mg of **ingredients** per kg
Matrices			**Thermal process**		**Thermal process**	**Thermal process**	**Thermal process**	**Thermal process**	-
			Cookie (180°C-18min), Sauce (95°C-45min), Chocolate, Ice cream		Bread (200°C -60 min)	Cookie (200°C -12 min)	Cookie (190°C -25 min)	Cookie (180°C -18 min), Bread (180°C - 40 min)	Chocolate
Purification			Sep-Pack C18 6cc (Waters)		Ultrafiltration (Amilcon Ultra 15 mL, 5 kDa molecular weight cut-off) (Millipore)	Size exclusion column (G25 Sephadex column)	Amicon Ultra 0,5 mL, Membrane Ultracel-10, PMNL 10 kD)(Millipore)	Amicon Ultra 0.5 mL centrifugal filters MWCO 10 kDa (Sigma-Aldrich)	SPE MonoSpin PBA spin column (GL-Science)
Instrument			Xevo TQS (Waters)		API 4000QTrap (Sciex)	Linear Ion Trap mass spectrometer Velos Pro (Thermo Fisher Scientific)	QTRAP 6500 (Sciex)	QTRAP 6500 (Sciex)	QTRAP 6500 (Sciex)

***Table 8**: Sensitivity (LOD or LOQ) (expressed in mg (soluble) proteins or ingredients per kg food product) of mass-spectrometry-based methods for detecting several allergens in highly processed (bread and cookie), slightly processed (sauce), and/or incurred matrices (chocolate and ice cream). The sensitivities were compared to VITAL thresholds and classified as: lower than or equal to the VITAL threshold (green), slightly higher (orange), or much higher (red). Percentages of proteins were obtained from VITAL and/or NIST standards* (Heick et al., 2011; Pilolli et al., 2017; Planque et al., 2017 a; Boo et al., 2018; Gu et al., 2018; New et al., 2018).

Moreover, the sensitivity reached by these methods applied to processed foods is thus lower than that of most ELISAs, especially for egg white, which is highly impacted by thermal processing. Since few years, the National Institute of Standards and Technology produces standard material for food allergen analysis. However, only the percentages of total proteins are indicated, while the percentages of each protein should be indicated in order to apply conversion factor and to compare batch to batch. In the **Table 8,** most of the methods were developed for a single food matrix analysis or similar matrices (e.g. cookies and bread). The additional value of the method we developed is the analysis of target peptides in four different food products, which guarantees the possibility to detect peptides in a longer list of diversified food products. However, while the method was developed for four processed or complex food products, one should bear in mind that allergen detection is complicated by the fact that hundreds of food products have to be analyzed. The diversity of matrices and industrial processes makes it hard to detect an allergen at a single LOQ. For example, a decrease in sensitivity has been observed when analyzing products with a high fat or spice content. SMPR 2016.002, providing guidelines for the development of mass spectrometry methods, is headed in this direction by proposing a list of food products by allergen in which the method should be tested (Paez et al., 2016).

Developed methods are more and more sensitive and laboratories start to develop methods in processed food products, which is an important step toward the improvement of food labeling and the protection of allergic populations. To date, ELISA, PCR or mass spectrometry methods cannot guarantee allergen that products are allergen-free in all kind of food matrices. However, the rapid improvement in food allergen detection methods and the appearance of legal thresholds that could be imposed in the future should contribute to reach this goal soon.

I.IV Validation of the method

Our developed method has been accredited at CER Groupe in 2016 and is currently used as a confirmatory method or as an alternative to the ELISA approach. The UHPLC-MS/MS method is particularly useful when an ELISA fails to detect a target allergen despite the addition of target allergen standards. This is notably the case of milk detection in tomato concentrate, turmeric, olive tapenade, and spinach. To avoid false negatives, standard addition should be done systematically when detecting antigens by UHPLC-MS/MS or by ELISA.

The method was validated for the detection of milk, egg, soy and peanut peptides in cookies (180 °C-18 min), chocolate, ice cream and tomato sauce (95 °C-45 min). Labeled internal standards were added in order to control the retention time of the target peptides TANELNLLIL[$^{13}C_6{}^{15}N$]R (peanut), FFVAPFPEVFGK[$^{13}C_6{}^{15}N_2$] (milk), GGLEPINF[D_5]QTAADQAR (egg), and EAFGV[D_8]NMQIVR (soy). The specificity of the method was controlled by analyzing 12 allergen-free matrices (3 replicates per matrices). The sensitivity was validated by analyzing 12 incurred samples prepared at the LOQ, a S/N ratio higher than 10 and 3 for the first and the second transitions was fixed, respectively. Moreover,

the linearity of milk, egg, soy and peanut peptides was controlled. The repeatability of the method was also evaluated by analyzing 6 technical replicates within a day.

The robustness of an analytical procedure could be defined as " *a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage such as ability to reproduce the method in different laboratories or under different circumstances"* (Vander Heyden et al., 2001). The robustness of the analytical method was tested using four different analysts and also different food preparations (e.g. incurred food products prepared by different analysts and cooked in different ovens).

As discussed in the book chapter, the lack of harmonized guideline makes difficult the validation of methods for the detection of peptides by mass spectrometry. In connection with the harmonization, it is of greater importance that a guideline gives a complete validation procedure and performance criteria for the detection of allergens by mass spectrometry.

II Quantification of allergens by mass spectrometry

At CER Groupe, allergens are quantified by ELISA with a single calibration curve obtained with the matrix "speculoos". Our main objective was to quantify allergens using a single calibration curve for allergens in solvent or a matched or similar matrix. In mass spectrometry, two main strategies are used to quantify allergens in food matrices: label-free and labeled stable isotope quantification (Planque et al., 2017 c). The label-free quantification strategy is based on comparing the peptide signal intensities of different samples using an external calibration curve or standard addition (adding standards directly to the samples) (Montowska et al., 2017; Pilolli et al., 2018). The second strategy is based on the use of isotope-labelled (^{13}C-, ^{15}N-, D-labeled) peptides or proteins in order to correct the peak area corresponding to the unlabeled (light) peptide with that corresponding to the labeled (heavy) peptide (Brun et al., 2007; Chen et al., 2015 a; Huschek et al., 2016; Groves et al., 2018; Sayers et al., 2018). Several quantification strategies were tested in order to find the most readily applicable by routine laboratories.

Two different types of labeled peptides were identified as internal standards: labeled peptides and long labeled milk-egg and peanut-soy peptides.

II.I labeled peptides

On the basis of the sensitivity achieved with peptides in processed and incurred food matrices (cookies, sauce, ice cream, and chocolate), labeled peptides were synthesized. For labeling, we selected the most abundant and specific peptide of milk, egg, soy, and peanut.

Labeled peptides were synthesized by Eurogentec. An amino acid in each target peptide was labeled with deuterium or with nitrogen 15 and carbon 13. This yielded TANELNLLIL[$^{13}C_6{}^{15}N$]R (peanut), FFVAPFPEVFGK[$^{13}C_6{}^{15}N_2$] (milk), GGLEPINF[D_5]QTAADQAR (egg), and EAFGV[D_8]NMQIVR (soy). A mass difference of at least 5 between the light (unlabeled) and heavy (labeled) peptides was chosen given the unit resolution of the triple quadrupole mass spectrometer (Hoofnagle et al., 2015).

The labeled peptides were incorporated as internal standards into the sample before the extraction step in order to correct for the entire protocol. Three matrices (sauce, cookie, and ice cream) were spiked prior to extraction with increasing concentrations of milk, egg, soy, and peanut protein extracts and fixed concentrations of labeled peptides. After correcting the area of the "light peptide" peak with that of the "heavy peptide" peak area, a calibration curve was drawn for each peptide in each of the three matrices. As the calibration curves did not coincide, however, we concluded that the labeled peptides failed to correct for the entire protocol. After further investigation, we showed that the extraction/digestion steps were not corrected and that these steps were crucial to reaching the main goal of using a single calibration curve to quantify an allergen in various foodstuffs.

In order to correct the extraction and digestion steps, we used long labeled peptides combining milk with egg peptides and soy with peanut peptides, showing promising results for the quantification of β-casein in baked food spiked with the milk allergen (Chen et al., 2015 a). These long-labeled peptides were synthesized by Eurogentec. Two amino acids were added at each end of each long peptide in order to provide better correction of the digestion step. An amino acid of each target peptide was labeled with nitrogen 15 and carbon 13. This yielded: GRFFV[$^{13}C_5{}^{15}N$]APFPEVFGKGGL[$^{13}C_6{}^{15}N$]EPINFQTAADQARGS (milk-egg), and GREAFGV[$^{13}C_5{}^{15}N$] NMQIVRTANEL[$^{13}C_6{}^{15}N$]NLLILRGS (soy-peanut).

The use of long labeled peptides rather than labeled peptides allowed spiking the samples with both internal standards prior to extraction. Eight matrices (ham, paprika, sauce, mayonnaise, cookie, chocolate, jam, and compote) were spiked with increasing concentrations of milk, egg, soy, and peanut protein extracts and a fixed concentration of each labeled peptide. Unfortunately, the calibration curves obtained for each allergen in eight matrices did not coincide, despite correction with both labeled peptides. Use of the long-labeled peptides thus did not allow correcting, as expected, for the extraction/digestion steps. Consequently, for the moment it is not possible to use a single calibration curve for quantification of all the tested allergens.

II.II Standard addition combined with labeled peptides as internal standards

One strategy for allergen quantification in food samples is standard addition. This strategy consists in adding increasing amounts of allergens to the sample in order to draw a calibration curve and to determine the initial concentration of target allergen. This strategy was chosen because of the conclusion reached in the previous section. It allows confirming allergen detection at the LOQ and determining the amount of allergen in the sample. Recoveries were determined on spiked samples (LOQ and 10xLOQ) of the eight target matrices. The recoveries were compared with the specifications of AOAC guideline SMPR 2016.002, allowing a recovery from 60 to 120%. Internal standards were introduced before extraction, and after correction with internal standards, recoveries within the specified range were achieved: 65% without correction, 81.7% with labeled peptide correction (IS1), and 83.3% with long labeled peptide correction (IS2). For the quantification of peanuts in incurred chocolate containing 0, 2, 4, 10, or 30 mg peanut proteins per kg, peanut proteins were spiked at only two different concentrations 2.5 mg and 25 mg of peanut proteins pet kg (LOQ, 10x LOQ). Recoveries

from 50 to 93% were found with isotope labeled peptide correction (IS1) and from 47% to 87% with long labeled internal standard correction (IS2).

With this method and for the quantification of four allergens in eight matrices at the LOQ and at 10 x LOQ, more than 80% of recoveries fell within the range specified by SMPR 2016.002 [60-120%]. As shown in **Table 9**, the proportion of out-of-specified-range recoveries was higher at the LOQ, suggesting that the range should be adapted as in guideline SANTE/11813/2017 for pesticide analysis and in regulation 2002/657/EC for the analysis of veterinary drug residues (Direction 2002/657/EC, 2002; SANTE/11813/2017, 2017). In **Table 9**, the recoveries obtained with our method are compared with those achieved by the most suitable state-of-the-art methods. To date, among the published quantification strategies directly applicable in routine laboratories for the quantification of several allergens in several food matrices, none gives recoveries strictly within the range 60 to 120%.

As shown in the **Table 9**, different calibration curves were used: standard addition (spikes in the targeted samples), matrix-matches calibration either with spiked samples, incurred samples or digested matrix spiked with digested proteins or incurred food matrices. Calibration curve based on digested matrix and digested proteins generates a bias due to the lack extraction and digestion of proteins in the target matrix, leading to weak recoveries in some matrices. As matrix effect was shown during method testing, matrix-matched calibration implies that a different calibration curve should be applied for each targeted sample. Finally, the use of incurred calibration curve is unrealistic as the laboratories should also have access to blank samples (allergen-free matrices), have good knowledge about process conditions of the product and prepare incurred materials for the establishment/generation of calibration curves (homogeneity test)

Authors	Allergens	Peptides	Internal standard (IS)	Matrices	Calibration curve	Concentration	Recovery			RSD (%)
								LOQ	10 x LOQ	
Chapter V (manuscript under submission)	Milk (αS1-Casein)	FFVAPFPEVFGK	**Labeled peptides IS 1**: FFVAPFPEVFGK [13C615N2], EAFGV[D8]NMQIVR, TANELNLLIL[13C6 15N]R, GGLEPINF[D5]QTAADQAR **Long labeled peptides IS2:** GRFFV[13C515N]APFPEVFGKGGL[13C615N]EPINFQTA AD QARGS, GREAFGV[13C515N]NMQIVRT ANEL[13C615N]NLLILRGS	Cookie, chocolate, jam, spice, compote, chicken ham, sauce and mayonnaise	**Standard addition** 6 levels of concentration + labeled peptides	**Spiked samples** at the LOQ: 0.5 mg for milk, 0.75 mg for egg, 2.5 mg for peanut and 5 mg for soy per kg and 10 x LOQ. **RSD** (n=3, LOQ x 10 in compote)	**IS1**	Milk: 59.3 - 133.5%	92.9 - 109.1%	5%
							IS2	37.5 -108.1%	58.2 - 120.9%	4%
	Egg (white)	GGLEPINFQTAADQAR					**IS1**	Egg: 85.7 - 196.6%	41.1 - 121.6%	4%
							IS2	90.6 - 128.3%	47.5 - 119.8%	8%
	Soy	EAFGVNMQIVR					**IS1**	Soy: 75 - 194.1%	90.3 - 108.7%	4%
							IS2	62.7 - 136.2%	75.4 -111.6 %	1%
	Peanut	TANELNLLILR					**IS1**	Peanut: 87.5 - 157.5%	74.7 - 111.6%	1%
							IS2	69 - 121.7%	78.6 - 116.8%	1%
Zhang et al. (2012)	Milk (α lactalbumin)	VGINYWLAHK	**synthetic internal standard** KILDKVGINNYWLAHKALCSE	infant formula matrix	**Standard addition** 4 levels of concentration (0, 1, 3 and 5 g/100g) α lactalbumin + IS peptide VGINNYWLAHK	**Fortified samples** at 10 000 mg of α lactalbumin per kg	Milk: 95.8 - 100.6%			< 6%
Pilolli et al (2018)	Egg	GGLEPINFQTAADQAR	No internal standard	Cookie	**Spiked samples** 300 and 600 mg of ingredients per kg	**Fortified samples** at 300 and 600 mg of ingredients per kg	Egg: 51 - 95%			<7%
		LTEWTSSNVMEER								
		YPILPEYLQCVK								
	Milk	TPEVDDEALEK					Milk: 55 - 69.8%			<50%
		YLGYLEQLLR								
		FFVAPFPEVFGK								
	Soy	ESYFVDAQPK					Soy: 63.4 - 70.9%			<10%
		SQSDNFEYVSFK								
		FYLAGNQEQEFLK								
	Peanut	AMVIVVVNK					Peanut: 59 - 68%			<10%
		GTGNLELVAVR								
		EGEQEWGTPGSEVR								
		VLLEENAGGEQEER								
	Hazelnut	ADIYTEQVGR					Hazelnut: 52 - 60%			<17%
		TNDNAQISPLAGR								
		QGQVLTIPQNFAVAK								
		ALPDDVLANAFQISR								
New et al. (2018)	Milk	YLGYLEQLLR	YLGYLEQL[13C615N4]R	Cookie, cookie dough, bread, ice cream, milk chocolate, breakfast cereal, dark chocolate, red and white wine, infant formula, and salad	**Digested matrices spiked with digested proteins** at 10, 100 and 1000 mg of allergens per kg	**Fortified samples**: cookie et bread and **spiked samples** for the rest of matrices 10, 100 and 1000 mg of allergens per kg. **RSD** (n=7)	Milk: 30.8 - 119.3 %			< 10.3%
	Egg (white)	GGLEPINFQTAADQAR	GGLEPINFQTAADQA[13C615N4]R				Egg: 39.9 - 117.5 %			< 16.2%
	Egg (yolk)	LPLSLPVGPR	LPLSLPVGP[13C615N4]R				Egg: 59.6 - 113.3%			< 12.2%
	Peanut	WLGLSAEYGNLYR	WLGLSAEYGNLY[13C615N4]R				Peanut: 16.7 - 110.6 %			< 10.2%
	Hazelnut	ADIYTEQVGR	ADIYTEQVG[13C615N4]R				Hazelnut: 5.8 - 77.6%			<12.9%
Gu et al. (2018)	Milk	FFVAPFPEVFGK	No internal standard	Choocolate	**External matrix-matched calibration curves**: fortified chocolate (8 concentrations)	**Fortified chocolate** at 4, 8 and 20 mg/kg for milk protein, 8, 16 and 40 mg/kg for peanut, hazelnut, cashew, and pistachio protein, and 12, 24 and 60 mg/kg for soybean, walnut, and almond protein in chocolate. **RSD** (n=3)	Milk: 62.4 - 70.2%			N.D
		NAVPITPTLNR					71.3 - 85.6%			
		VLPVPQK					61.5 - 92.4%			
		YIPIQYVLSR					73.1 - 88.1%			
		QQQEEQPLEVR					62.6 - 88.8%			
	Soy	TISSEDEPFNLR					Soy: 60.1 - 81.2%			
		VLIVPQNFVVAAR					62.4 - 85.8%			
	Peanut	GTGNLELVAVR					Peanut: 61.2 - 80.1%			
		RPFYSNAPQEIFIQQGR					60.9 - 78.1%			
	Almond	GNLDFVQPPR					Almond: 65.4 - 83.8%			
		ADFYNPQGGR					70.1 - 86.0%			
	Walnut	VFSNDILVAALNTPR					Walnut: 72.9 - 88.2%			
	Hazelnut	ADIYTEQVGR					Hazelnut: 72.5 - 85.6%			
	Cashew	ADIYTPEVGR					Cashew: 77.7 - 87.5%			
	Pistachio	EGQLVVVPQNFAVVK					Pistachio: 65.3 - 85.7%			

Table 9*: Recovery and relative standard deviation (RSD) obtained with mass-spectrometry-based methods for quantifying several allergens. In spiked samples , extracts of allergens proteins are added to the sample, while in fortified samples, raw ingredients are added to the sample both after the process* (Zhang et al., 2012; Gu et al., 2018; New et al., 2018; Pilolli et al., 2018)

Finally, in the absence of a method allowing the quantification of peptides in food products using a single calibration curve, the standard addition with protein extracts seems to be the best approach for the quantification of peptides in food products. Indeed, the standard addition ensures the detection of peptides at the LOQ and should be able to correct the matrix effects observed in apparently "similar matrices" such as, for example, different red wines.

III Prospects

III.I Detection of allergens

Selection of marker peptides: In this project, allergens were incorporated in several processed and complex matrices, but a single reference standard was used (for example, prepared with NIST 1549a milk powder). Meanwhile, another study is being carried out to produce standard materials for milk, egg, hazelnut, and peanut (raw peanuts from different countries, peanut milk or roasted peanuts). The main objective of that project is to identify, by high-resolution mass spectrometry (Q-Tof), marker peptides common and specific to each production process for allergens used in manufacture.

Cleaning - Currently, cleaning of the UHPLC column is performed between samples in order to avoid contamination, but detecting allergens in samples such as yoghurt, having a high percentage of milk, is still impossible because of major sample carry-over (contamination causing sample peaks to re-appear in subsequent runs) after several cleanings. Column cleaning to remove carry-over will constitute a major improvement of the method. To date, we recommend the use of a specific column for the analysis of allergens in milk products to avoid contaminations or the improvement of cleaning to avoid carry-over.

Implementation - The method is currently implemented for the detection of gluten and lupine, both viewed as major allergens in the European Union (Regulation No. 1169/2011, 2011). In the future, the method will be extended to the remaining allergens (sesame, mustard, celery, macadamia nut, Brazil nut, fish, crustaceans, and mollusks).

Field - The method has also been used for sensitive detection of bovine blood meal, blood products, and milk products in aqua feed (Lecrenier et al., 2018). The official method for detecting blood products in aqua feed is PCR, but this technique cannot distinguish bovine blood, prohibited since the mad cow crisis, from milk products, which are allowed. This is why a UHPLC-MS/MS method has been developed to distinguish milk from bovine blood with high specificity. Consequently, the method could be also used for the detection of peptides in other areas of expertise.

Purification - SPE purification has enabled us to achieve good sensitivity for the ten target allergens, but immunoaffinity purification should be investigated further in order to allow specific purification of proteins and perhaps to improve the sensitivity of the method for egg, almond, walnut, and pecan in order to reach VITAL thresholds (i.e. VITAL: 0.75 mg egg proteins per kg instead of the 3 mg egg proteins per kg reached by our method). The SPE MonoSpin PBA spin column (GL-Science) used by Gu et al. could be also tested in order to avoid possible contamination of the SPE system caused by highly contaminated samples (Gu et al., 2018).

III.II Quantification of allergens

III.II.1 Labeled proteins

The principle of this approach is to add a labeled protein having the same structure than the native protein to the sample before extraction. The labeled proteins should allow correcting for the matrix effect and for effects linked to different steps in the sample preparation protocol (protein extraction and enzymatic digestion). To date, only Newsome et al. studied the recovery of the milk allergen α-S1 casein in baked cookies using a ^{15}N-labelled α-S1 casein, and obtained recoveries ranging from 60 to 80% for the quantification of milk (Newsome et al., 2013).

An extension of the present work is embodied in a recently launched food allergen project. Its participants have adopted the so-called "gold standard" strategy, i. e. the use of labeled proteins as internal standards, to achieve the goal that we pursued here: a single calibration curve for the quantification of allergens by mass spectrometry. They have chosen the protein to be labeled on the basis of two criteria: the number of target peptides in the protein and the number of amino acids (so as to limit the price of synthesis). The chosen protein is a milk protein: β-lactoglobulin, composed of 162 amino acids. Recombinant β-lactoglobulin is synthesized by ProteoGenix in France. The unlabeled protein will be produced first in order to optimize the method and to determine the yield of synthesis, after which the labeled protein will be produced.

To avoid false positives due to incomplete labeling, targeted labeling of specific amino acids will be performed. Three amino acids, previously tested by the supplier for the synthesis of labeled proteins, could be labeled: leucine (L), lysine (K), and methionine (M). In our method, two peptides of β-lactoglobulin are analyzed: VLVLDTDYK and LSFNPTQLEEQCHI, both of which contain two leucines and have a charge state of 2. Hence, the amino acid leucine has been chosen for labeling. As ^{15}N-labeling of the peptide backbone of a protein at selected residues is often more rapid and more reliable than ^{13}C-labeling, the project participants have chosen to label a leucine with ^{15}N (Waugh, 1996). The protein will be synthesized by the prokaryote *Escherichia coli*, a host cell widely used for the production of recombinant proteins notably because of its short generation time, high yields, and relatively low cost (Rosano et al., 2014; Zheng et al., 2014; Jia et al., 2016).

The labeled proteins and the protein will be introduced before extraction and then subjected to enzymatic digestion, the resulting labeled (heavy) peptide and unlabeled (light) peptide will be analyzed by UHPLC-MS/MS. As triple quadrupoles have unit resolution, they might not distinguish a light and a heavy peptide differing by an m/z of 1. Should they fail to do so, the strategy will be tested and validated with an instrument affording better resolution, and the strategy will then be adapted to a lower-resolution instrument (for example, by labeling two amino acids, which would of course be costlier). Several matrices will be spiked with milk and the labeled protein prior to extraction and digestion, and the peak area corresponding to the light peptide will be corrected by the peak area corresponding to the heavy peptide in order to determine if a single calibration curve can be used. This should offer the advantage of being more applicable in routine laboratories than the standard addition strategy previously developed. It should perfectly correct for the extraction and digestion

steps, unlike long labeled peptides, allowing better recoveries for the quantification of allergens. Unfortunately, the cost of labeled proteins decreases the feasibility of this strategy in routine laboratories.

III.II.2 Concatemeric proteins

The use of one labeled protein per allergen will be extremely costly, so another strategy will also be tested for the quantification of allergens in food products. This strategy is based on the use of internal standards (called QconCAT proteins) consisting of concatenated tryptic peptides containing different ^{15}N- or ^{13}C-labeled peptides of the target proteins (Beynon et al., 2005). The QconCAT proteins will be tested along with the labeled proteins to achieve the goal that we pursued here: a single calibration curve for the quantification of allergens by mass spectrometry. One should note, however, that producing a QconCAT protein from recombinant DNA can increase the formation of aggregates, limiting the yield of properly folded proteins, and this might compromise the use of such proteins as standards (Nilsson et al., 2005; Brownridge et al., 2011). Moreover, the structure of the QconCAT protein will be different, and this might hinder the digestion of some peptides or even make it hard to extract the QconCAT protein from food matrices. This would lead to inadequate correction of matrix effects and of the different analytical protocol steps. To avoid poor digestion of peptides in the QconCAT protein, the predicted protein will be analyzed to determine if undesirable secondary structure features are likely to be present (Pratt et al., 2006).

For each protein of interest, one or several peptides will be selected for the synthesis of QconCAT proteins, as shown in **Figure 51**.

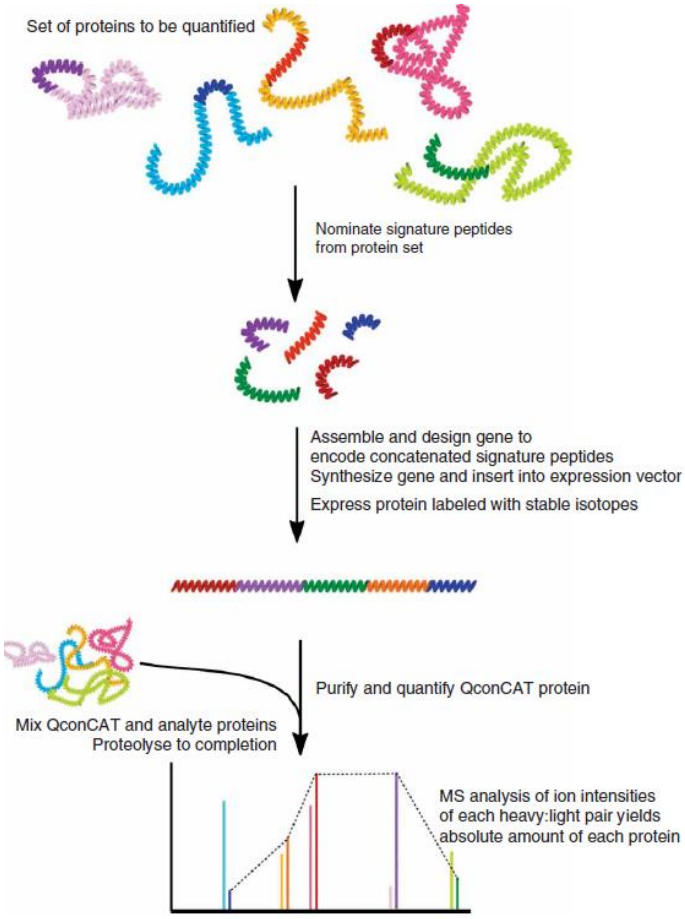


Figure 51*: Principle of QconCAT quantification: (1) The peptide sequence is concatenated in silico in order to design a gene that expresses this artificial protein. (2) E. coli cells will be transformed with the plasmid, and after a period of exponential growth, QconCAT synthesis will be induced. (3) The QconCAT protein will be digested with trypsin and peptides will be analyzed by high resolution mass spectrometry in order to check the sequence of the protein and check for the absence of unlabeled peptides* (Pratt et al., 2006; Rivers et al., 2007; Brownridge et al., 2011)*. From* (Pratt et al., 2006).

In conclusion, these two strategies seem promising. They could allow reaching our goal of a single calibration curve and improving current recoveries for allergen quantification.

Even though developed methods are increasingly tested in several matrices (processed and unprocessed) and allow sensitive detection of several allergens, one should point out that there are still many challenges to be met, mostly in terms of harmonization (reference standards and materials, internal standards, reporting units…) and allergen quantification, to achieve reliable analysis of allergens. Food laboratories still have a long way to go to provide standard methods allowing reliable allergen labeling and to ensure protection of the allergic population.

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ANNEX 1: LABINFO N16 OF THE FEDERAL AGENCY FOR THE SAFETY OF THE FOOD CHAIN (FASFC)

Food allergen analyses: state of the art and perspectives for food control

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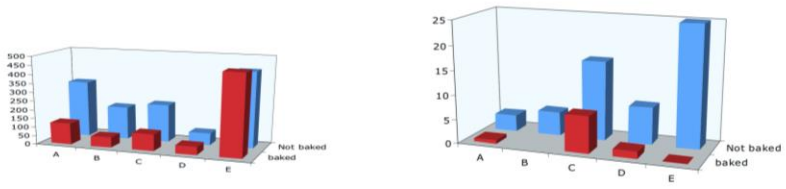
Food allergy is defined as an adverse immunologic response to a particular food ingredient. The reactions and severity vary from patient to patient and are associated with a broad array of signs and symptoms, the severest being anaphylactic shock [1]. With a rising pattern in prevalence, current estimates show that nearly 5% of the adults and at least 8% of children are affected by food allergies [2]. Although numerous clinical studies have been conducted, the only current prevention strategy relies on the elimination of the provocative allergenic ingredient from the patients diet. To help allergic customers, European Directive EC/1169/2011 requires the labelling of the 14 most important allergens on food products if used as ingredient or products thereof [3,4]. However, food products can be contaminated during food production with unwanted food allergens which can also cause severe allergic reactions [5]. To protect allergic customers, food manufactures make use of precautionary allergen labelling (PAL), such as "may contain …". Because there is a lack of relation between PAL labelling and allergen risk assessment, it has become untrustworthy and sometimes unnecessarily to restrict the diet of the patient [6].

In order to protect the allergic consumers and to avoid expensive recalls, reliable food allergen detection methods are of utmost importance. However, food control laboratories have to face some difficulties which are described in this article.

1/ Available methods for food allergen control

The most widespread used methods are based on recognition of allergen proteins by antibodies, like lateral flow devices methods (food industries) and enzyme-linked immunosorbent assay methods (ELISA) (routine laboratories). However, modification of allergenic proteins by food processing, or presence of interfering compounds (polyphenols, high fat content,…) can compromise the binding of antibodies. This could lead to underestimations and even false negative results, as shown on Figure 1 [7–10]; or, in contrast, binding of antibodies to other related/homologues proteins may lead to false positive results, also known as cross-reactivity problems.

Figure 1: Analytical results for 1000 mg/kg of milk powder (National Institute of Standards and Technology SRM 1549a) baked or not baked cookies obtained with different ELISA kits for (A) casein and (B) β-lactoglobulin detection [7]
[x-axis: ELISA kits; y-axis: estimated concentration (unit of the kit)]



DNA-based methods, like real-time polymerase chain reaction (PCR), are also used to detect the presence of the allergenic commodity. This technique proves the presence of the ingredient, but the presence of an ingredient specific DNA fragment does not prove the presence of the allergenic protein. Sometimes, it can lead to false-positive results (e. g. confusion between chicken and egg DNA) [11]. However, such a cross reactivity is also observed for ELISA tests where e.g. kits detecting mustard will also give cross reaction with rapeseed [12].

The need for reliable analytical methods for food allergen testing has prompted researchers to develop mass-spectrometry-based methods as additional tools [13]. Ultra-high performance liquid chromatography coupled to tandem mass spectrometry (UHPLC-MS/MS) is based on the analysis of peptides after an enzymatic digestion [14]. Despite the high investment cost and need for trained staff, UHPLC-MS/MS methods have the advantages to target several allergens in a single analysis (Fig. 2), and to be highly specific and sensitive, even for the analysis of processed foodstuffs.

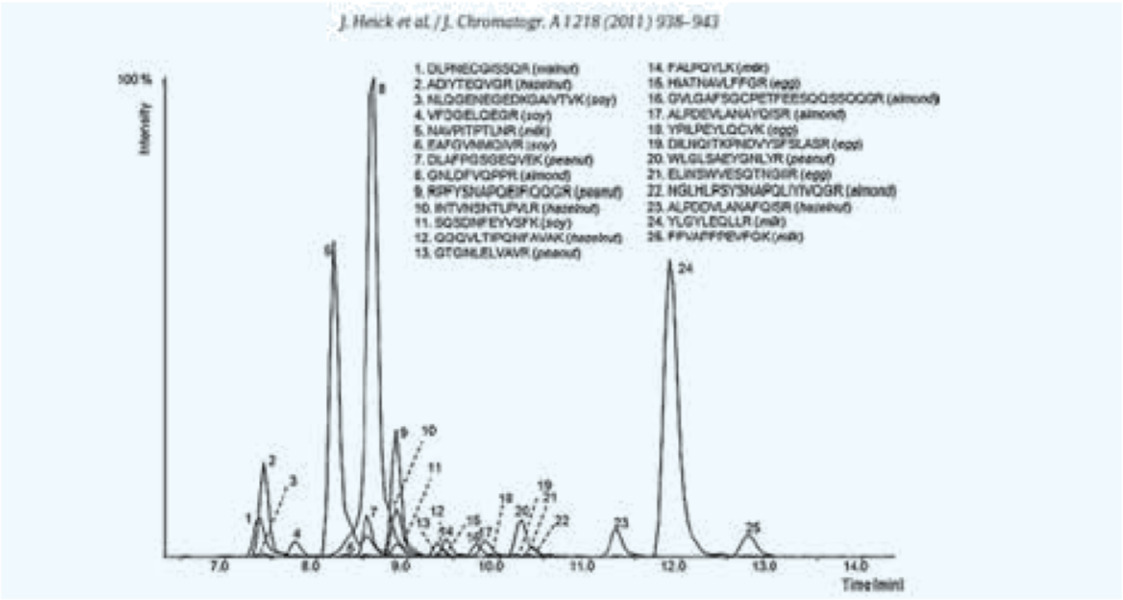


Figure 2: MRM (Multiple Reaction Monitoring) transitions for the incurred bread reference material containing 1000 mg/kg of all seven allergenic foods [15]

2- Food allergens: remaining challenges

Even if food industries and food authorities have currently access to several available techniques for food allergens testing, laboratories still have to face some challenges.

2.1 Lack of regulatory threshold

To face this lack, the Voluntary Incidental Trace of Allergen Labelling (VITAL) system was developed in Australia and New-Zealand to help food producers in the management of cross-contaminations during food production. VITAL fixed global thresholds for raw and baked allergens, based on clinical data [16,17]. Unfortunately, this system does not have any regulatory value, while several national Food Authorities often refer to it during risk assessment.

2.2 Lack of method harmonization

2.2.1 Difference between reporting units

Food laboratories developed DNA-, protein- or peptide-based methods for allergens testing, nevertheless food industries or authorities have to make a choice between these approaches or a combination of them. The lack of common reference standards, as well as discrepancies in reporting units, makes the comparison of results difficult, even between one method type as shown in Table 1.

Table 1: Comparison of some standards used by ELISA providers.

Provider	Format	Standard	Sensitivity threshold (LOQ) (mg/kg)	Range of quantification (mg/kg)
Kit 1	Sandwich	caseins	0,5	0,5 -20
Kit 2	Sandwich	skim milk powder	1,0	1,0 – 10,0
Kit 3	Competitif	whole milk powder NIST SRM 8435 (expressed in casein)	1,6	1,6 – 25
Kit 4	Sandwich	milk protein	0,312	0,312 – 20

A common strategy should be adopted; this could include conversion factors and/or even better the use of reference materials.

2.2.2 Determination of method sensitivity

The determination of method sensitivity is evaluated through different approaches: addition of protein extract [21,22] (in buffer or in food products), or allergen contaminations in food products before/after cooking [18,20,23].

ELISA's sensitivities are often estimated by spiking food matrices, after production, with allergen extracts. However, to ensure effectiveness of the detection method, it is becoming more common to assess sensitivity – for example during validation of UHPLC-MS/MS methods (table 2 [15,18–20]) - using feed matrices which were spiked with allergenic ingredient before preparation. This strategy allows a more realistic estimation of method sensitivity regarding real food products analyses.

Table 2: Determination of method sensitivity by different UHPLC-MS/MS methods in baked food products compared with VITAL thresholds for a portion size of 40 g of food products.

	Milk	Egg	Peanut	Soybean	Expression of results
VITAL	**2,5**	**0,7**	**5**	**25**	**mg of total proteins by kg**
Heick *et al.* [15]	10	50	10	50	mg of soluble proteins by kg
Gomaa *et al.* [18]	10	-	-	10	mg of total proteins by kg
Monaci *et al.* [19]	26	30	30	18	mg of ingredients by kg
Planque *et al.* [20]	0.5	3,4	2,5	5	mg of total proteins by kg

This leads to huge differences in estimated method sensitivity (limit of quantification (LOQ)). An example of LOQ variability through these different approaches is presented in Figure 3.

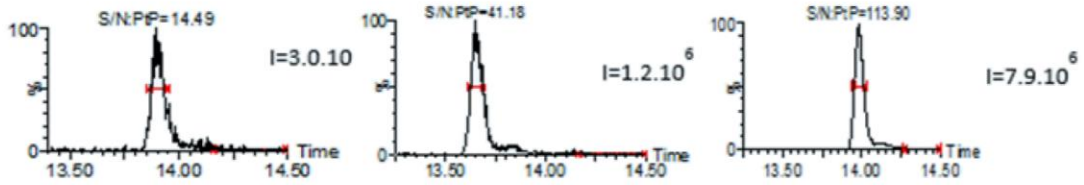


Figure 3: chromatograms of milk casein αS1 peptide FFVAPFPEVFGK (692.7>920.5) obtained for 0.5 mg of milk proteins by kg of (A) baked cookies (180°C -36 min) theoretical sensitivity threshold ratio signal to noise (S/N=10) 0.5 mg/kg, (B) addition of protein extract in cookie theoretical sensitivity threshold 0.125 mg/kg and (C) addition of protein extract in solvent theoretical sensitivity threshold 0.05 mg/kg. Milk powder: NIST 1549a (25.6% of proteins) were used to perform this experiment following the protocol describe in the publication: Planque et al. 2016 [20].

The difference between these chromatograms proved the necessity to follow a common guideline on method performance criteria (such as described in [24]), as well as the need for reference standards and materials to reach methods harmonization.

2.3 "Fit-for-purpose" analytical methods

Routine laboratories have to face another challenge: the wide variety of food sample types received for analysis of food allergens. Every method cannot be validated for all sample types, while routine analytical service should guarantee trueness and precision of the results for all samples, even in presence of interferences or in baked foodstuffs. The scope of an analytical method should therefore be clearly defined. Laboratories also have to design a strategy to quantitatively validate their method, especially for processed foodstuffs.

3/ Conclusion

The consumers are looking for safe food products. Food producers are therefore pushed to abandon abusive precautionary allergen labelling, and replace it with decent food allergens risk assessment/management. To achieve this, reliable analytical methods need to be used. And in turn, evolution of the used methods already led to improved analytical sensitivity. A real improvement was done for allergen detection in baked food products with UHPLC-MS/MS methods, with a sensitivity close to or lower than the threshold level fixed by the VITAL system. However, there is still a long way to go for method harmonization.

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ANNEX 2: NEWSLETTER AOAC FOOD ALLERGEN COMMUNITY

NEWSLETTER
Volume 7 | Issue 3 2016

Major challenges for reliable detection of allergens in food products ■

Food allergies have increased impressively over the last few decades. In order to protect customers with food allergies, allergens must appear clearly on food labels. Labelling food products adequately can be hard, however, because of potential cross-contaminations during food production. To help food producers in this tedious task, the food community has developed analytical methods for food allergen detection. Nonetheless, there remain issues that must be addressed in order to harmonize food allergen control.

Recently, although the number of food allergen analyses has increased dramatically, regulatory thresholds still have to be established. Systems proposing thresholds in food products (VITAL, EAACI…) have been developed, but these thresholds have not yet acquired regulatory force.

Method sensitivities are mostly determined by spiking solvents or matrix extracts with allergens. This approach makes methods attractive, but creates a significant gap between declared and real thresholds, especially for heat-processed food products. Some laboratories, aware of this shortcoming, have produced and commercialized incurred materials (FAPAS, MoniQA, LGC…). Another problem is that sensitivity determinations based on measurements in only one matrix cannot guarantee sensitive detection of allergens in processed matrices or in ones with a high fat content or high acidity. That a method is fit for purpose should thus be established carefully in each case.

Despite the emergence of incurred and standard materials (from producers such as the National Institute of Standards Technology and LGC Standards), there is a lack of standards for most allergens. Unfortunately, this leads to the use of different materials (walnuts, roasted walnuts, walnut milk…). Furthermore, different members of the food community report their results in different units: ingredients, soluble proteins (BCA quantification), total proteins (theoretical content), or protein (casein, ovalbumin…). The lack of standards and guidelines makes it very hard to compare methods, so that results are almost useless for laboratories and even worse for food producers. Concerned about this lack of harmonization, the AOAC Stakeholder Panel on Strategic Food Analytical Methods (SPSFAM) has recently established requirements for the evaluation of mass-spectrometry-based methods for allergens (SMPR 2016.002 "Standard method performance requirements for detection and quantification of selected food allergens"). In its guidelines, this panel recommends reference standards, target matrices, ranges of quantification, etc.

The publication of validation guidelines and the development of reference standards and materials are first steps towards harmonization and method performance testing. Yet the food community still has a long way to go for full harmonization of methods.

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Summary of the lack of harmonization encountered by the food community and possible solutions.

Identified problems	Expression of results	Ingredients	Proteins		Protein
			Soluble proteins (BCA quantification)	Total proteins (Theoretical content estimation)	Casein αS1, β-lactoglobulin
	Determination of method sensitivity	Spiked		Incurred	
Solutions	Solution for harmonization	Reference materials and standards Regulatory Thresholds	Regulatory thresholds	Guidelines Mass Spectrometry methods (SMPR 2016.002)	